

POH TING FUNG

**ANGIOTENSIN II- AND VASOPEPTIDASE- INHIBITORY
ACTIVITIES OF GYNURA PROCUMBENS**

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**FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR**

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INHIBITORY ACTIVITIES OF
GYNURA PROCUMBENS

POH TING FUNG

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ABSTRACT

Blood pressure (BP) is partly determined by vasoconstriction or vasodilation. The most important vasoconstrictor is angiotensin II (Ang II) whilst nitric oxide (NO), prostacyclin (PGI₂) and bradykinin (BK) are main vasodilators. The vasopeptidase, angiotensin-converting enzyme (ACE), is able to hydrolyse BK and convert angiotensin I (Ang I) to Ang II. Bradykinin is also inactivated by another vasopeptidase, neutral endopeptidase (NEP). Increases in ACE and NEP activities would elevate Ang II but decrease BK levels that could cause increases in BP which in the long-term could result in hypertension.

Many antihypertensive drugs are available but with side-effects. Alternative forms of treatments, especially those from the plant kingdom, are frequently sought after. *Gynura procumbens* (*G. procumbens*) is widely used in folk medicine to treat hypertension. Previous findings show that leaves of this plant seem to decrease BP by inhibiting ACE activity and blocking calcium channels. The aims of this project are to further study the BP-lowering properties of this plant by exploring the Ang II and ACE/NEP inhibitory activities of a partially purified fraction (FA-I) and to characterise the bioactive subfractions.

Endothelium-intact and -denuded rat aortic rings suspended in organ chambers were used to study the effects of FA-I on Ang I- and Ang II-induced contractions. The role of endothelium on the effects of FA-I was tested further by adding N^ω-nitro-L-arginine methyl ester (L-NAME) or indomethacin to ascertain the involvement of NO and PGI₂ respectively. Effects of FA-I on the vasodilatory response due to BK were studied by *in vitro* and *in vivo* methods. Rats were also given oral doses of FA-I to examine the stability of the fraction against digestive enzyme degradation. The FA-I fraction was subjected to further separation by thin layer chromatography (TLC) to obtain the FA-I subfractions, the

activities of which on ACE/NEP were analysed by spectrophotometric and spectrofluorometric assays. After ^1H -nuclear magnetic resonance analysis, one of the bioactive subfractions was sent for further structural characterisations.

Results show that FA-I significantly ($p < 0.05$) decreased the contraction evoked by Ang I and Ang II in endothelium-intact and -denuded rings with the degree of relaxation being significantly ($p < 0.05$) higher in intact ones. However, in the presence of L-NAME or indomethacin, the inhibitory effect of FA-I on Ang II-induced contraction was significantly ($p < 0.05$) reduced. In contrast, FA-I enhanced the vasorelaxant effects of BK, which may contribute to the greater BP-lowering effects of BK. Activities of ACE and NEP was inhibited by FA-I. Early-eluted subfractions obtained from TLC exhibited specific inhibition on ACE alone. Study also shows that the ACE inhibitory activity of FA-I was still preserved after the actions of proteases and spectral analyses showed that FA-I subfractions may contain 16-carbon compounds.

In conclusion, FA-I was found to be able to inhibit Ang II-induced contractions, probably via endothelium-dependent pathways by activating NO or PGI_2 release, and also of being able to inhibit ACE/NEP activities. Nuclear magnetic resonance and mass spectroscopic analyses indicate that one of the subfractions (FA-Ia) could be a 16-carbon compound but the precise structure remains to be elucidated.

ABSTRAK

Tekanan darah (BP) ditentukan secara separa oleh aktiviti vasokonstriksi atau vasodilatasi. Angiotensin II merupakan vasokonstriksi yang terpenting manakala nitrit oksida (NO), prostaglandin I_2 (PGI_2) dan bradikinin (BK) adalah vasodilatasi utama. Vasopectidase, enzim pengubah angiotensin (ACE), mampu menyahaktif BK serta menukar angiotensin I (Ang I) kepada Ang II. Bradikinin juga dinyahaktif oleh satu lagi vasopectidase, neutral endopeptidase (NEP). Peningkatan dalam aktiviti ACE dan NEP yang meningkatkan Ang II tetapi mengurangkan tahap BK boleh menyebabkan peningkatan BP yang dalam jangka masa panjang mengakibatkan hipertensi.

Beberapa jenis ubat kini boleh didapati untuk mengawal hipertensi tetapi adalah dengan kesan sampingan. Alternatif rawatan, terutamanya daripada alam tumbuhan sering dicari. *Gynura procumbens* (*G. procumbens*) merupakan salah satu tumbuhan yang digunakan secara tradisi untuk merawat hipertensi. Penemuan sebelum ini telah menunjukkan bahawa daun daripada tumbuhan ini mengenakan kesan antihipertensi seolah-olahnya melalui aktiviti perencatan ACE dan penyekatan saluran kalsium. Jadi, projek ini bertujuan untuk terus mengkaji sifat penurunan tekanan darah tumbuhan ini dengan menerokai aktiviti perencatan Ang II dan ACE/NEP oleh fraksi akueus *G. procumbens* yang separa tulen (FA-I) dan mencirikan subfraksi FA-I yang mempunyai bioaktiviti.

Cincin aorta tikus dengan endotelium-tergantungan dan -tidak tergantung yang dipasang dalam kamar organ telah digunakan untuk mengkaji kesan FA-I terhadap vasokonstriksi yang disebabkan oleh Ang I dan Ang II. Peranan endotelium dalam kesan FA-I seterusnya diuji dengan menambah N^{ω} -nitro-L-arginina methyl ester (L-NAME) atau indomethacin untuk memerhatikan penglibatan NO dan PGI_2 masing-masing. Kesan FA-I terhadap vasodilatasi yang dibawa oleh BK dikaji dengan kaedah *in vitro* dan *in vivo*.

Tikus juga disuap dengan FA-I untuk memeriksa kestabilan fraksi ini terhadap kemusnahan yang dibawa oleh enzim pencernaan. Fraksi FA-I ditakluk kepada pemisahan lagi oleh lapisan nipis kromatografi (TLC) untuk mendapatkan subfraksi FA-I, di mana aktiviti-aktiviti perencatan ACE/NEP dianalisis oleh spektrofotometer dan spektrofluorometer. Selepas analisis dengan ^1H -resonans magnetik nuklear, salah satu subfraksi dihantar selanjutnya untuk pencirian struktur molekulnya.

Keputusan menunjukkan bahawa FA-I dengan ketaranya ($p < 0.05$) menurunkan vasokonstriksi oleh Ang I dan Ang II dalam cincin endotelium-tergantung dan -tidak tergantung di mana vasodilatasi lebih tinggi di endotelium-tergantung. Walau bagaimanapun, dengan penambahan L-NAME atau indomethacin, kesan FA-I yang melarang vasokonstriksi oleh Ang II telah berkurang dengan ketara ($p < 0.05$). Sementara itu, FA-I meningkatkan kesan vasodilatasi BK yang mungkin mengakibatkan penurunan BP yang lebih kuat. Kesan penurunan tekanan darah oleh FA-I masih jelas walaupun selepas suapan menunjukkan bahawa aktiviti FA-I dapat menahani kesan enzim pencernaan. Tambahan pula, FA-I merencat aktiviti ACE dan NEP. Dalam subfraksi-subfraksi yang didapati, hanya subfraksi yang didapati lebih awal oleh TLC memamerkan aktiviti perencatan yang spesifik pada ACE. Baccan juga menunjukkan FA-I masih mengekalkan aktiviti perencatan ACE selepas tindakan oleh protease dan analisis spektra menunjukkan bahawa subfraksi FA-I mungkin mengandungi kompaun 16-karbon.

Sebagai kesimpulannya, FA-I didapati berupaya mengurangkan vasokonstriksi yang dibawa oleh Ang II dengan endotelium mungkin memainkan peranan untuk mengaktifkan rembesan NO dan PGI_2 di samping merencatkan aktiviti ACE/NEP. Selain itu, analisis spektroskopi resonans magnetik nuklear dan spektrometri jisim menunjukkan bahawa salah satu daripada subfraksi (FA-Ia) mungkin adalah kompaun 16-karbon tetapi struktur yang tepat masih belum dijelaskan.

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LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ACEI	Angiotensin-converting enzyme inhibitor
ACh	Acetylcholine
AMC	7-amino-4-methylcoumarin
APN	Leucine aminopeptidase
Ang I	Angiotensin I
Ang II	Angiotensin II
Ang III	Angiotensin III
Ang IV	Angiotensin IV
Ang (1-7)	Angiotensin (1-7)
Ang (1-9)	Angiotensin (1-9)
AngQb	CYT006-AngQb
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
ANS	Autonomic nervous system
APN	Leucine aminopeptidase
ATP	Adenosine triphosphate
AT ₁ R	Angiotensin II type 1 receptor
AT ₂ R	Angiotensin II type 2 receptor
AT ₄ R	Angiotensin type 4 receptor
B : A : W	<i>n</i> -butanol : acetic acid : distilled water
BB	Beta blocker
BK	Bradykinin
BP	Blood pressure

B ₁	Bradykinin type 1
B ₂	Bradykinin type 2
Ca ²⁺	Calcium ion
CaCl ₂	Calcium chloride
CAM	Complementary and alternative medicine
cAMP	Cyclic adenosine-3', 5-monophosphate
CCB	Calcium channel blocker
cGMP	Cyclic guanosine-3', 5-monophosphate
CO	Cardiac output
COX	Cyclooxygenase
CVD	Cardiovascular disease
D	Dimensional
DAGNPG	N-dansyl-Ala-Gly-D-nitro-Phe-Gly
DASH	Dietary approaches to stop hypertension
DBP	Diastolic blood pressure
DEPT	2D distortionless enhancement by polarisation transfer
EDH	Endothelium-dependent hyperpolarisation
eNOS	Endothelial nitric oxide synthase
ET	Endothelin
ET-1	Endothelin-1
FA	Final aqueous fraction
FA-I	Purer final aqueous fraction
FDA	Food and Drug Administration
FT	Fourier transform
gACE	Germinal ACE

GC	Guanylate cyclase
<i>G. procumbens</i>	<i>Gynura procumbens</i>
GTP	Guanosine triphosphate
H ₃ BO ₃	Boric acid
HA	Hippuric acid
HCl	Hydrochloric acid
HEPES	1,4-dioxan, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHL	Hippuryl-L-histidyl-L-leucine
HL	Histidyl-leucine
HMWK	High molecular weight kininogen
HPLC	High performance liquid chromatography
HR	Heart rate
JG	Juxtaglomerular
JNC 7	The seventh report of the joint national committee on prevention, detection, evaluation, and treatment of high blood pressure
KCl	Potassium chloride
K-H	Krebs-Henseleit
KH ₂ PO ₄	Potassium dihydrogen phosphate
L-NAME	N ^ω -nitro-L-arginine methyl ester
LMWK	Low molecular weight kininogen
MAP	Mean arterial pressure
MgSO ₄	Magnesium sulphate
MS	Mass spectrometry
MW	Molecular weight

NaCl	Sodium chloride
NaHCO ₃	Sodium bicarbonate
NaOH	Sodium hydroxide
NEP	Neutral endopeptidase
NEPI	Neutral endopeptidase inhibitor
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
NP	Natriuretic peptide
P-AMC	Phe-7-amino-4-methylcoumarin
PE	Phenylephrine
PGI ₂	Prostacyclin
RAS	Renin-angiotensin system
R _f	Retention factor
ROS	Reactive oxygen species
SAAP-AMC	Suc-L-Ala-L-Ala-Phe-7-amino-4-methylcoumarin
sACE	Somatic ACE
SARS	Severe acute respiratory syndrome
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive
SD	Sprague-Dawley
SV	Stroke volume
TCM	Traditional Chinese medicine
TLC	Thin layer chromatography

TPR	Total peripheral resistance
TXA ₂	Thromboxane A ₂
UV	Ultraviolet
VPI	Vasopeptidase inhibitor
VSM	Vascular smooth muscle
v/v	Volume/ volume
WHO	World Health Organisation
WKY	Wistar-Kyoto
w/v	Weight/ volume
Zn ²⁺	Zinc ion
[Ca ²⁺] _i	Intracellular calcium ion

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CHAPTER 1 - INTRODUCTION

1.1 Blood pressure

1.1.1 Regulation of blood pressure

Blood pressure (BP) is the force exerted by the blood against any unit area of the blood vessel wall (Guyton and Hall, 2006). The BP in humans is usually measured by the indirect auscultatory method and is expressed as systolic BP (SBP) over diastolic BP (DBP) (SBP/DBP) mmHg. In physiology, BP can be calculated from the formula: $BP = \text{cardiac output (CO)} \times \text{total peripheral resistance (TPR)}$ (Kirkman and Sawdon, 2010). Cardiac output is the volume of blood that is pumped out of the ventricle per minute and is expressed as $CO = \text{stroke volume (SV)} \times \text{heart rate (HR)}$ (Kirkman and Sawdon, 2010), whereas TPR refers to the total peripheral resistance of the entire systemic circulation. In CO, SV is the volume of blood that is pumped out of the ventricles during one heart beat whereas HR is the number of beats per minute. The SV is mainly controlled by venous return (preload), outflow resistance (afterload) and force of ventricular contractility (Ackermann, 2004; Wilcken, 2010). Increases in preload and ventricular contractility, and a decrease in afterload will increase the SV. As for the HR, it is mainly controlled by cardiac pacemaker cells that are increased by positive chronotropic agents (e.g. cardiac sympathetic nervous activity) and decreased by negative chronotropic agents (e.g. cardiac parasympathetic nervous activity) (Ackermann, 2004).

Resistance in blood vessels largely depends on smooth muscle activities that change the radius of the lumen, either by vasocontraction or vasodilation. Vascular smooth muscle (VSM) activities are affected by the endothelium, autonomic nervous system and blood borne substances such as adrenaline, acetylcholine, adenosine triphosphate (ATP) and substance P (Kirkman and Sawdon, 2010). Overall, any factor that influences SV, HR and TPR will change the BP (Kirkman and Sawdon, 2010).

In order to provide and maintain sufficient blood supply to organs and tissues, BP is regulated all the times, either by short-term or long-term mechanisms. Under resting conditions, arterial BP is controlled mainly by baroreceptors (Kirkman and Sawdon, 2010) via altering the cardiovascular parameters of SV, HR or TPR (Ackermann, 2004). In this short-term regulation of BP, the change in the stretch of arterial walls is rapidly sensed by baroreceptors located in the walls of aortic arch and carotid sinuses (Kirkman and Sawdon, 2010) that reflexedly activate or inhibit the sympathetic or parasympathetic division of the ANS and renin-angiotensin system (RAS). In the long-term regulation of BP, however, the kidney plays the most important role (Ackermann, 2004) by regulating the total body content of sodium, water and other electrolyte balance.

In addition, circadian rhythm also plays a key role in BP regulation. In a healthy person, the BP rises in the morning, followed by night time drop in a cycle that occurs once every 24 hours (Rudic and Fulton, 2009). This circadian rhythm may become abnormal during hypertension in which the person may become nondippers (absence of nocturnal BP drop), extreme dippers (marked nocturnal BP drop) or reverse dippers (rise in nocturnal BP) (Rudic and Fulton, 2009).

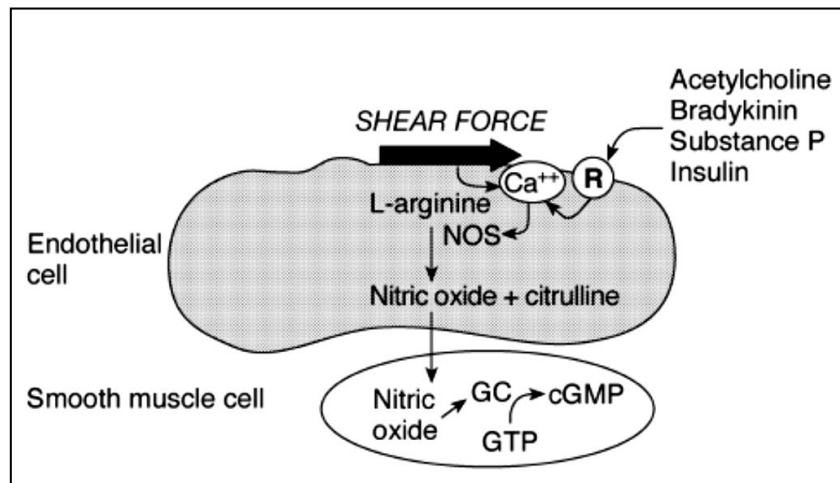
1.1.2 Endothelium

The endothelium is a monolayer of cells lining the entire inner layer of blood vessels. This layer represents an important component in inflammation, platelet aggregation, angiogenesis, endocrine functions (Feletou, 2011) and the regulation of vascular contractility by releasing vasoactive substances that change the degree of contraction of the underlying VSM (Vanhoutte and Mombouli, 1996). The vasoactive substances produced by the endothelium are vasodilators such as nitric oxide (NO) and prostacyclin (PGI₂) that cause endothelium-dependent hyperpolarisation (EDH) (Feletou

and Vanhoutte, 2013) or vasoconstrictors such as endothelin-1 (ET-1) and thromboxane A₂ (TXA₂) that presumably result in membrane depolarisation (Sandoo *et al*, 2010). Another powerful vasoconstrictor is that of angiotensin II (Ang II) and it is discussed in the RAS section (page 11).

1.1.2.1 Nitric oxide

Nitric oxide, first identified by Furchgott and Zawadzki in 1980 (Furchgott and Zawadzki, 1980) is an important component in the control of basal vasodilator tone in blood vessels (Vallance *et al*, 1989). This compound is formed from L-arginine by the action of NO synthase (NOS), an enzyme that exists in three isoforms: neuronal NOS, macrophage or inducible NOS and endothelial NOS (eNOS) (Vanhoutte and Mombouli, 1996). Of these, eNOS appears to play a main role in dilation of blood vessels (Sandoo *et al*, 2010). Figure 1.1 summarises the formation of NO by activation of eNOS. Stimuli such as shear stress and the binding of NO agonists such as bradykinin (BK), acetylcholine (ACh), insulin or substance P to respective receptors increase intracellular calcium ion (Ca²⁺) ([Ca²⁺]_i) and cause calmodulin in the endothelial cells to bind to eNOS that then converts L-arginine to NO. The released NO then diffuses into smooth muscle cells and activates soluble guanylate cyclase (GC), which converts guanosine triphosphate (GTP) to cyclic guanosin-3', 5-monophosphate (cGMP) (Sandoo *et al*, 2010). Subsequently, a protein kinase is activated by cGMP to inhibit calcium influx into the smooth muscle cells which then decreases the contraction of smooth muscle cells to cause vasodilation (Galley and Webster, 2004).



NOTES: NOS = nitric oxide synthase; GC = guanylate cyclase; GTP = Guanosine triphosphate; cGMP = cyclic guanosin-3', 5-monophosphate

Figure 1.1 : Schematic representation of the activation of endothelial nitric oxide synthase that finally produces nitric oxide to cause vasodilation (*reproduced with permission*). (adapted from Galley and Webster, 2004)

1.1.2.2 Prostacyclin and thromboxane A₂

Although both PGI₂ and TXA₂ are prostanoids, they have opposing effects on VSM in that PGI₂ is a vasodilator whereas TXA₂ is a vasoconstrictor. The enzyme that is involved in the formation of PGI₂ and TXA₂ is cyclooxygenase (COX), wherein two isoforms of COX: COX-1 which is expressed in endothelial cells and other tissues; and COX-2 which is expressed during endothelial damage or in the presence of various cytokines (Feletou, 2011). Both isoforms are involved in the synthesis of PGI₂ and TXA₂ and the synthesis of either depends on the types of tissues and on whether there is inflammation or other influences (Feletou, 2011).

In the normal healthy condition or in the presence of stimuli such as oxidative stress, PGI₂ is constitutively produced in the endothelial cells from prostaglandin H₂ by the actions of prostacyclin synthase (Tang and Vanhoutte, 2008). The PGI₂ then binds to its receptor (IP receptor) to activate adenylate cyclase that induces the production of cyclic adenosine-

3', 5-monophosphate (cAMP) (Vanhoutte and Mombouli, 1996) to cause vasodilation (Fetalvero *et al*, 2007).

Thromboxane A_2 is also produced from prostaglandin H_2 , by the actions of thromboxane A synthase (Yokoyama *et al*, 1991). In healthy VSM or presence of physical stimuli such as stretch, this unstable compound with a short half-life (about 30 seconds) binds to its receptor (TP receptor) to cause platelet aggregation, angiogenesis, and endothelium-dependent contractions by increasing $[Ca^{2+}]_i$ (Nakahata, 2008).

1.1.2.3 Endothelin-1

Endothelin (ET) is a strong vasoconstrictor and exists in three isoforms: ET-1, ET-2 and ET-3 (Inoue *et al*, 1989). Endothelin-1, the only isoform produced in vascular endothelium (Masaki, 2004), is formed from big ET-1 by the action of endothelin-converting enzyme, a zinc-metalloproteinase that is anchored to the plasma membrane (Yanagisawa *et al*, 1988).

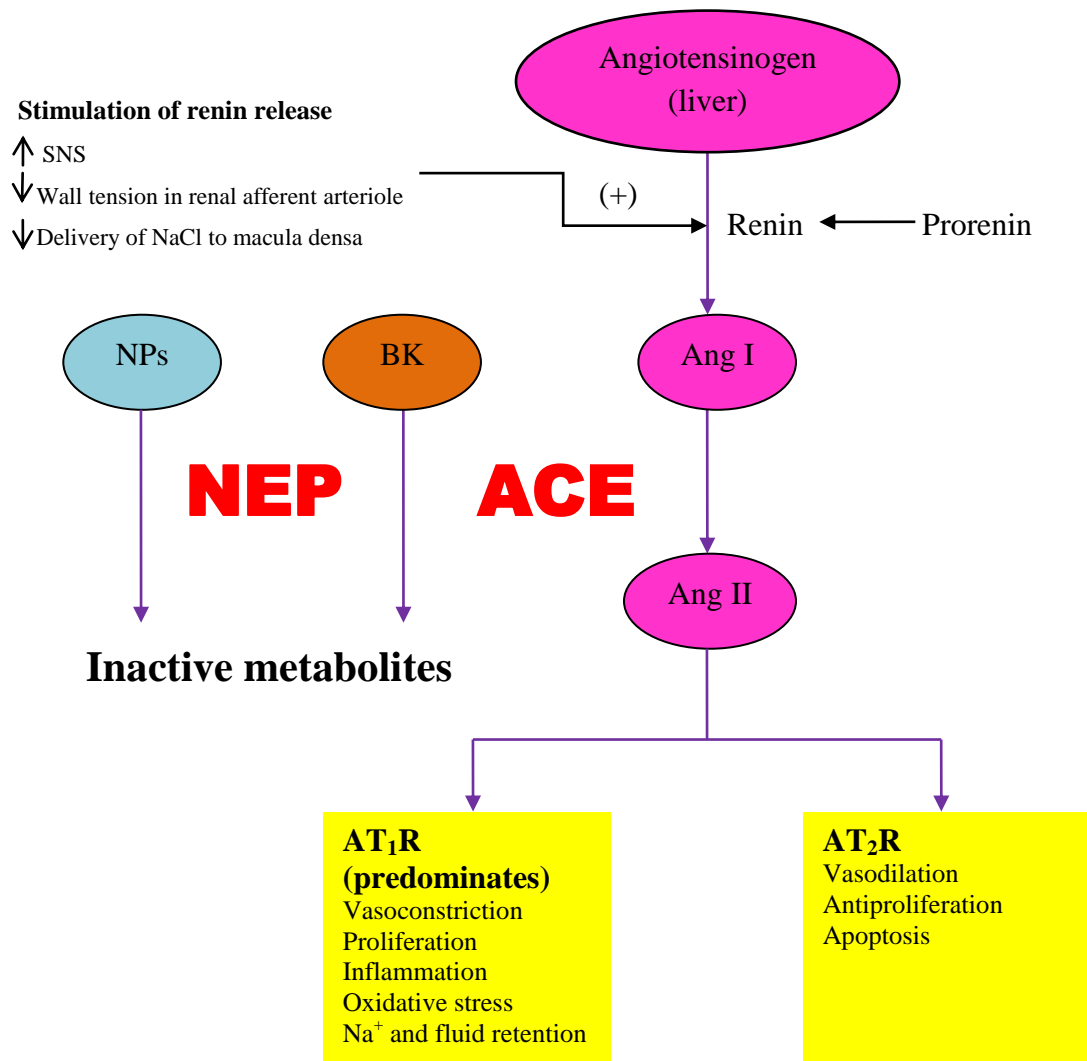
In tissues, two types of ET-1 receptors are found: ET_A and ET_B (Luscher, 1994). Endothelin-1 binds to these receptors, predominantly ET_A (Luscher, 1994) to increase $[Ca^{2+}]_i$ and cause vasocontraction (Zhang¹, 2000). However, under certain conditions, ET-1 can cause vasodilation via the activation of ET_B receptor, by stimulating the release of NO (Cardillo *et al*, 2000).

1.2 Renin-angiotensin system

1.2.1 Overview

The main homeostatic mechanism in the body that is involved in the regulation of BP, as well as extracellular fluid volume and sodium content balance is RAS (Lote, 2006). In the RAS, the key enzyme renin, is synthesised in an inactive form as ‘prorenin’ in the granular cells of the afferent arterioles in the juxtaglomerular (JG) cells of the kidneys (Guyton and Hall, 2006). Renin splits 10-amino acid fragments from angiotensinogen that is produced in the liver to form the decapeptide angiotensin I (Ang I) which has no physiological effect (Hayashi and Kimoto, 2010) and within seconds to minutes, it is cleaved of two amino acids to form octapeptide Ang II by an enzyme called angiotensin-converting enzyme (ACE) (Guyton and Hall, 2006; Lote, 2006). Although the Ang II persists in the blood for 1-2 minutes only, it accounts for the main function of RAS to restore the BP to the normal level (Guyton and Hall, 2006).

The RAS is involved in both physiological and pathological processes. In the physiological processes, RAS takes part in development, learning and memory as well as tissue growth. However, in pathological process, RAS plays a role in the progression of disorders such as diabetes, hypertension, cardiovascular disease (CVD) and also tumors (Nguyen, 2006; Atlas, 2007). The roles and characteristics of renin, Ang II, ACE and the roles of receptors for angiotensin II are shown in Figure 1.2.



NOTES: SNS = Sympathetic nervous system; Ang = Angiotensin; ACE = Angiotensin converting enzyme; BK = Bradykinin; NPs = Natriuretic peptides; NEP = Neutral endopeptidase; AT₁R, AT₂R = Angiotensin type 1, 2 receptor;

Figure 1.2 : Activation of renin-angiotensin system and the effects on related receptors as well as the actions of neutral endopeptidase (adapted from Ferrario and Iyer, 1998; Turner and Hooper, 2002; Carey and Park, 2006; Hunyady and Catt, 2006; Atlas, 2007; Daull *et al*, 2007; Lambert *et al*, 2008; Harrison-Bernard, 2009; Benigni *et al*, 2010).

1.2.2 Renin

Renin is the key regulatory enzyme in the RAS (Sequeira Lopez and Gomez, 2010) and it exists in plasma in two forms: inactive prorenin (about 90%) and mature renin (Nguyen, 2006). The inactive prorenin becomes mature renin in the JG cells of the kidneys, which is then released into the plasma when triggered by the three main mechanisms that are increased sympathetic activity to the granular cells of the afferent arterioles, decreased tension in the walls of the afferent arterioles and decreased delivery of sodium chloride to the macula densa (Lote, 2006). In addition, renin expressing cells are restricted to JG cells only (Sequeira Lopex *et al*, 2004). However, in conditions such as hemorrhage that triggers the homeostasis response, there is an increase in renin expressing cells outside the JG cells such as in renal vascular smooth muscle cells and glomerular cells (Sequeira Lopex *et al*, 2004). This is useful in order to produce enough renin to reestablish homeostasis (Sequeira Lopex *et al*, 2004).

1.2.3 Angiotensin-converting enzyme

Angiotensin-converting enzyme, a zinc ion (Zn^{2+})-dependent dipeptidyl carboxypeptidase and an ectoenzyme, is anchored to cell membranes by C-terminal (Shen *et al*, 2008). It exists as two isoforms: somatic ACE (sACE) (Bernstein *et al*, 1988) which is found in many tissues such as vascular endothelia (Ryan *et al*, 1976), endothelial lining of lungs (Turner and Hooper, 2002), brush borders of kidney and intestine (Cushman and Cheung, 1971); and the testicular or germinal ACE (gACE) isoform which is located only in testes (Bernstein *et al*, 1989). Somatic ACE has two active sites with two domains (C- and N-domains) whereas gACE has only one active site (C-domain), which is identical to the C-domain of sACE (Ehlers *et al*, 1989).

In general, ACE functions by hydrolysing Ang I, BK, substance P and cholecystokinin (Turner and Hooper, 2002) as well as cleaving Ang (1-7) (Chappell *et al*, 1998). Both the domains of sACE are believed to have distinct functions wherein the C-domain is involved in RAS regulation and BK hydrolysis, whereas the N-domain is connected with the processing of bioactive peptides and regulation of renal structure and functions (Coates, 2003). In contrast, the C-domain in gACE is involved in the sperm maturation process (Metayer *et al*, 2002; Coates, 2003). Nevertheless, these two isoforms of ACE cannot act as a substitute for each other in their respective physiological functions (Kessler *et al*, 2007).

1.2.4 Angiotensin-converting enzyme 2

A homologue for ACE that shares 42% of amino acid sequence with ACE is ACE 2. It is less distributed and restricted to the heart, kidney and testes (Donoghue *et al*, 2000). There are differences in the structures and actions of ACE homologues in which ACE is a dipeptidyl carboxypeptidase that cleaves dipeptides from substrate whereas ACE 2 is a carboxypeptidase that cleaves only one single amino acid from its substrate (Guy *et al*, 2005; Lambert *et al*, 2008).

In the RAS, ACE 2 cleaves Ang I to form angiotensin (1-9) [Ang (1-9)] and also splits Ang II to angiotensin (1-7) [Ang (1-7)], a compound which is becoming popular for their protective role in RAS. The Ang (1-9) is also converted by another ectoenzyme, the neutral endopeptidase 24.11 (NEP), and also by ACE to form Ang (1-7) (Welches *et al*, 1993). Studies have shown that Ang (1-7) potentiates the effect of BK (Greco *et al*, 2006), stimulates NO and prostaglandin release (Rajendran, Chirkov *et al*. 2005) but antagonises the effect of Ang II (Grobe *et al*, 2007). The affinity of ACE 2 for Ang I is weaker when compared to ACE and the imbalance in the level of ACE and ACE 2 in the body is believed

to be responsible for CVD and also hypertension (Der Sarkissian *et al*, 2006). Other than that, a report shows that ACE 2 is a receptor for severe acute respiratory syndrome (SARS) coronavirus (Li *et al*, 2003).

1.2.5 Neutral endopeptidase

Another Zn^{2+} -dependent metalloendopeptidase that is also involved in BK metabolisms (Figure 1.2), is neutral endopeptidase (NEP), also known as enkephalinase, neprilysin, neutral metalloendopeptidase or common acute lymphoblastic leukemia antigen. Similar to ACE, NEP also exists in the membrane-bound form and it is widely distributed in kidneys, the central nervous system, lungs, male genital tract, intestines, and in neutrophils, fibroblasts and epithelial cells (Erdos and Skidgel, 1989).

Neutral endopeptidase metabolises vasoconstrictor peptides such as enkephalins and Ang II as well as the vasodilator peptides, BK, substance P and natriuretic peptide (NP) (Ruschitzka *et al*, 2001). The main effect of NEP is similar to ACE that is to increase BP. Although ACE is the main enzyme in the metabolism of BK, in human cardiac tissues, NEP is more important in metabolising BK (Kokkonen *et al*, 1999). The most important role of NEP is in the metabolism of NPs which present in three isoforms: the well-studied atrial-NP (ANP), brain-NP and C type-NP (Ruschitzka *et al*, 2001). The ANP reduces BP by increasing natriuresis as well as inhibiting RAS, endothelin and angiogenesis (Ruschitzka *et al*, 2001; Sagnella, 2002).

1.2.6 Angiotensin II

Angiotensin II binds to two types of G protein-coupled receptors, known as Ang II type 1 receptor (AT₁R) and type 2 receptor (AT₂R) (Lote, 2006; Benigni *et al*, 2010). Angiotensin II type 1 receptor is widely distributed especially in VSM cells (Hunyady and Catt, 2006) and it mediates most of the actions of Ang II (Lote, 2006) which vary at different effector tissues (Peach and Dostal, 1990). For instance, Ang II is a vasoconstrictor in systemic circulation (Peach and Dostal, 1990) but has both vasodilator and vasoconstrictor effects in cerebral circulation (Maktabi *et al*, 1990). Nevertheless, the overall main effect of Ang II is to increase BP by causing vasocontraction and this contraction is a fast reaction and occurs rapidly after Ang II binding to AT₁R (Touyz and Schiffrin, 2000). Increased production of Ang II due to increased RAS activity is considered one of the many contributing factors for the onset of hypertension (Navar *et al*, 2011). Table 1.1 summarises the effects brought about by activation of AT₁R by Ang II.

In contrast, the level of AT₂R is low in healthy adults but only exists at high level in fetal tissues (Ichiki *et al*, 1995) and pathological states such as heart failure (Liu *et al*, 1997) and myocardial infarction (Searles and Harrison, 1999). This receptor may take part in brain development as well (Gendron *et al*, 2003). Stimulation of AT₂R by Ang II antagonises the effects brought by AT₁R activation (Benigni *et al*, 2010) and also activates the BK type 2 (B₂) receptor to cause vasodilation which is likely to be mediated by the NO pathway and cGMP cell signalling cascade (Searles and Harrison, 1999; Carey and Park, 2006).

Table 1.1 : Effects of angiotensin II type 1 receptor activation.

-
- Vasoconstriction
 - Stimulation of thirst
 - Release of antidiuretic hormone and aldosterone
 - Increased sodium reabsorption by direct effects on the proximal tubule of the nephron, effect of aldosterone on distal tubules and also increase in renal sympathetic nerve activity
 - Angiogenesis, cellular growth and hypertrophy
 - Induction of production of reactive oxygen species (ROS)
 - Increase in the production of vascular endothelial growth factor in the inflammatory process
-

(Adapted from Searles and Harrison, 1999; Hunyady and Catt, 2006; Lote, 2006; Benigni *et al*, 2010)

Angiotensin II has a short half-life of about one minute (Lote, 2006). It is cleaved by aminopeptidase A to angiotensin III (Ang III) which has the same affinity for AT₁R and AT₂R. Angiotensin III plays a main role in the regulation of brain RAS as well as stimulating the release of aldosterone. It is then cleaved by aminopeptidase N to angiotensin IV (Ang IV) which has low affinity for AT₁R and AT₂R (Hunyady and Catt, 2006) but a higher affinity for angiotensin type 4 receptor (AT₄R) to increase natriuresis and renal blood flow. Angiotensin IV is also involved in memory and learning process (Turner and Hooper, 2002).

1.2.7 Non angiotensin-converting enzyme dependent angiotensin II formation

Angiotensin II can also be formed from non-ACE dependent pathway and the enzyme responsible for this is chymase (Huang *et al*, 2003). Chymase, a chymotrypsin-like serine protease found in the mast cells (Takai *et al*, 1999), is thought to be the main enzyme for Ang II formation in human hearts (Urata *et al*, 1990), arteries (Huang *et al*, 2003) and the kidneys (Huang *et al*, 2003). This pathway provides a need to suppress the non ACE-dependent Ang II formation in order to fully suppress the RAS (Petrie *et al*, 2001).

1.3 Kallikrein-kinin system

1.3.1 Overview

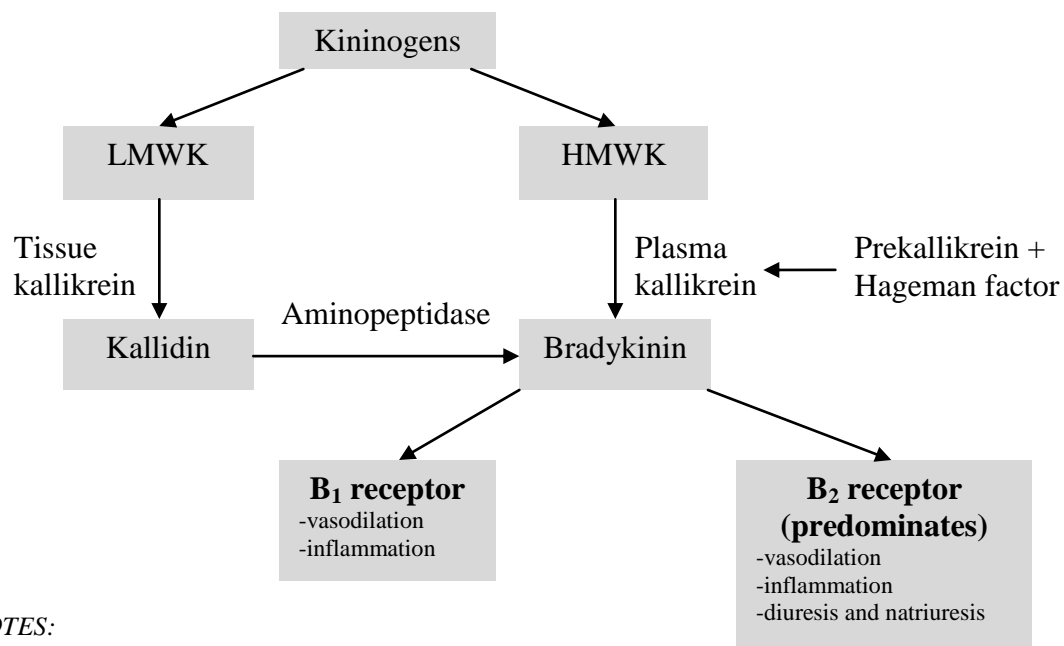
The kallikrein-kinin system is a complex system that has a close relationship with the RAS in the regulation of BP. There are three main constituents in the kallikrein-kinin system: kallikreins, kininogens and kinins (Campbell, 2000). Kallikreins are serine proteases and can be classified into tissue kallikrein and plasma kallikrein. Kininogens are proteins of two types: low molecular weight kininogen (LMWK) and high molecular weight kininogen (HMWK). The kallikrein-kinin system produces two main kinins which are BK and kallidin.

The kallikrein-kinin system is predominantly found in tissues rather than in the circulation (Golias *et al*, 2007). As shown in Figure 1.3, the precursor for plasma kallikrein is prekallikrein that forms a complex with Factor XII (Hageman Factor). When there is tissue damage, prekallikrein is changed to plasma kallikrein that then converts HMWK to BK while tissue kallikrein hydrolyses LMWK to kallidin that will eventually be converted to BK by aminopeptidase (Campbell, 2000).

There are two types of G-coupled protein receptors for BK, known as BK type 1 (B₁) and B₂ receptors. The B₂ receptor predominates under healthy conditions whereas the B₁

receptor is induced by tissues injuries such as myocardial ischemia and inflammation and also by endogenous factors such as growth factors, endotoxins and cytokines (Campbell, 2000; Sharma and Al-Sherif, 2011). When the B₁ receptor is activated, it causes vasodilation and initiates the inflammatory response (Sharma and Al-Sherif, 2011). As for B₂ receptor, it has higher affinity for BK (Leeb-Lundberg *et al*, 2005) and activation of B₂ receptor causes vasodilation by releasing EDH mediators and cGMP from NO (Vanhoutte, 2001). Figure 1.3 shows the activation of kallikrein-kinin system that leads to the production of kallidin and BK as well as the actions of BK receptors.

Kinin receptors are also linked to many types of diseases such as CVD, renal disease, airway disease especially asthma (Barnes 1992), neurological disease, cancer as well as arthritis, hereditary angioedema and gastrointestinal disease (Leeb-Lundberg *et al*, 2005). Thus, kinin receptors are also a pharmacological target in treating these diseases.



NOTES:

LMWK = Low molecular weight kininogen;

HMWK = High molecular weight kininogen;

B₁R, B₂R = Bradykinin type 1, type 2 receptor

Figure 1.3 : Activation of kallikrein-kinin system that finally leads to activation of bradykinin receptors (B₁ and B₂) (adapted from Campbell, 2000 and Sharma and Al-Sherif, 2011).

1.3.2 Bradykinin

Bradykinin is important in the regulation of BP and pain and in the inflammatory process, and it is also said to have cardioprotective (Sharma and Al-Sherif, 2011), diuretic and natriuretic effect (Willis *et al*, 1969). Binding of BK to B₂ receptor increases local production of BK to induce endothelium dependent relaxation and the main action of BK is at the heart, kidney and blood vessels (Vanhoutte, 2001). In the inflammatory process, BK increases vascular permeability to fluid and plasma proteins that results in oedema (Sharma and Al-Sherif, 2011) while in the management of pain, BK is both algescic and hyperalgescic (Sharma and Al-Sherif, 2011).

Degradation of BK occurs either at its amino or carboxy terminal (Figure 1.4). Enzymes that cleave at amino terminal are aminopeptidase M and P whereas enzymes that are responsible for the degradation of BK at carboxy terminal are kininase I and kininase II

(Sharma and Al-Sherif, 2011). Kininase I includes carboxypeptidase M and N whereas kininase II are ACE and NEP (Dorer *et al*, 1974; Kokkonen *et al*, 1999; Sharma and Al-Sherif, 2011). Figure 1.4 shows the enzymes that degrades BK at its amino or carboxy terminal.

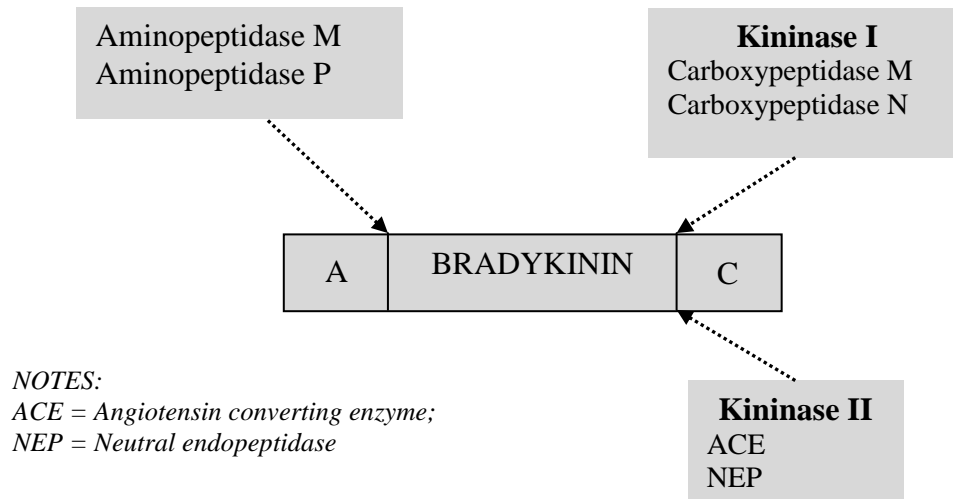


Figure 1.4 : Degradation of bradykinin that occurs at amino (A) and carboxy (C) terminals.

1.4 Hypertension

1.4.1 Overview

Hypertension or high BP occurs when the body fails to bring the BP back to the normal set-point. Hypertension is generally regarded as chronic elevation of BP $\geq 140/90$ mmHg. It is called ‘silent killer’ because it is asymptomatic and can cause severe health problems and even death (Rudic and Fulton, 2009). Hypertension is classified into primary or secondary hypertension. Primary, also well-known as essential or idiopathic hypertension accounts for 95% of all cases of hypertension and it is referred to the hypertensive condition in which secondary causes such as renal failure, pheochromocytoma and aldosteronism are absent (Carretero and Oparil, 2000). Secondary hypertension is the hypertension in which the underlying causes such as kidney diseases, sleep apnea, hormonal diseases and vascular diseases are known. According to The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC 7) (Chobanian *et al*, 2003), for adults 18 years old and older, their BP can be classified into normal, prehypertension and hypertension (stage 1 and stage 2) (Chobanian *et al*, 2003), as shown in Table 1.2.

However, the definition for hypertension by JNC 7 which is according to the BP threshold method lacks the ability to identify underlying CVDs in people with normal BP (Giles *et al*, 2005). Thus, a writing group within the American Society of Hypertension has produced a new definition of hypertension which takes into account different physiological abnormalities in the cardiovascular system as well as other organs caused by hypertension. They define hypertension as “a progressive cardiovascular syndrome arising from complex and interrelated etiologies. Early markers of the syndrome are often present before blood pressure elevation is observed; therefore, hypertension cannot be classified solely by discrete blood pressure thresholds. Progression is strongly associated with functional and

structural cardiac and vascular abnormalities that damage the heart, kidneys, brain, vasculature, and other organs, and lead to premature morbidity and death.” (Giles *et al*, 2005; Giles *et al*, 2009)

Table 1.2 : Classification of blood pressure for adults ≥ 18 years old according to The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (Chobanian *et al*, 2003).

BP Classification	SBP (mmHg)	DBP (mmHg)
Normal	<120	and <80
Prehypertension	120-139	or 80-89
Stage 1 hypertension	140-159	or 90-99
Stage 2 hypertension	≥ 160	or ≥ 100

Notes: BP = Blood pressure; SBP = Systolic BP; DBP = Diastolic BP

1.4.2 Global burden of hypertension

In 2000, more than 25% of the adult population in the world was estimated to have hypertension and it is predicted that 1.56 billion adult population will have hypertension by 2025 (Kearney *et al*, 2005; Kim *et al*, 2010). The overall prevalence of hypertension for subjects aged ≥ 18 years in the United States in 2003-2004 was 29.3% (Ong *et al*, 2007). In Malaysia, the prevalence of hypertension for subjects aged ≥ 15 years was 40.5% in 2004 (Rampal *et al*, 2008).

Hypertension is the most common risk factor for CVD and end organ damage which are the main cause for mortality and morbidity worldwide (Foex and Sear, 2004; Schmieder, 2010). According to JNC 7, individuals with BP between 130-139/85-89 mmHg have more than twice the risk of getting CVD than individuals with BP below 120/80 mmHg (Chobanian *et al*, 2003). Although the morbidity and mortality that linked

to hypertension is high, the control of BP to optimal level among hypertensive patients is less than 50% (Foex and Sear, 2004; Jackson *et al*, 2008; Nahas, 2008).

1.4.3 Risk factors for hypertension

Blood pressure is the product of CO and TPR which means that hypertensive patients may have increased CO, increased TPR or increased in both of these parameters (Foex and Sear, 2004). Increase in any of these parameters rise the BP. Table 1.3 shows some of the identifiable risk factors for hypertension.

Table 1.3 : Identifiable risk factors for hypertension.

Unchangable factors:

- Genetics (Lifton, 1995; Agarwal *et al*, 2005)
- Aging (Chobanian *et al*, 2003; Lee and Oh, 2010)
- Gender (Dubey *et al*, 2002)

Behavioral factors:

- High alcohol and salt intake (Carretero and Oparil, 2000)
- Stress (Dogru *et al*, 2010; Joyner *et al*, 2010)
- Sleep deprivation (Gangwisch *et al*, 2006)

Other factors:

- Obesity (Strazzullo *et al*, 2001; Beltowski, 2010)
 - Diabetes (Esler *et al*, 2006; Kotsis *et al*, 2010)
-

1.4.4 Treatments for hypertension

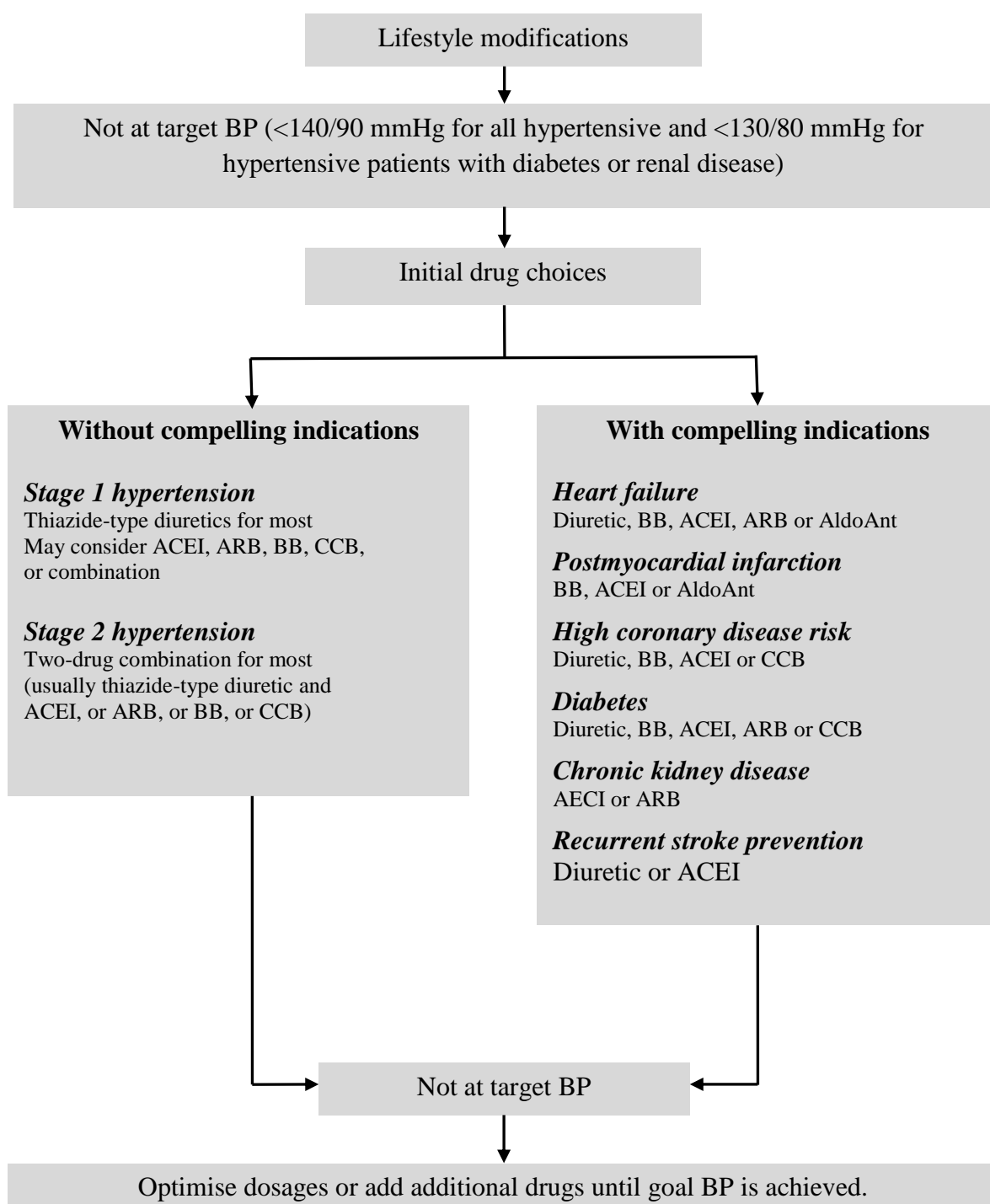
In general, the treatment goal in the control of BP for hypertensive patients is <140/90mmHg while the goal for hypertensive patients with diabetes or renal diseases is <130/80mmHg (Chobanian *et al*, 2003). The algorithm for the treatment of hypertension by JNC 7 is shown in Figure 1.5. According to JNC 7, the first line of treatment for hypertension is lifestyle modification which includes weight reduction, adoption of the Dietary Approaches to Stop Hypertension (DASH) eating plan, dietary sodium reduction, regular physical activity and moderation of alcohol consumption (Chobanian *et al*, 2003). The DASH eating plan includes diet high in vegetables, fruits, low-fat dairy products with smaller amount of red meat and sugar intake, and with decreased amount of total and saturated fat and cholesterol (Sacks *et al*, 2001). Combination of this eating plan and dietary sodium reduction has been proven to be effective in reducing BP (Sacks *et al*, 2001).

If lifestyle modification fails to control the BP at normal levels, pharmacological treatment will be considered by physicians. Choices of drugs rely on their BP lowering effects and also consideration of potential CVD risk (Gradman *et al*, 2010). Generally, there are five main classes of drugs that are popularly used in treating hypertension, and they are low-dose thiazide diuretic, beta blocker (BB), calcium channel blocker (CCB), ACE inhibitor (ACEI) and angiotensin receptor blocker (Hill and Smith, 2005). Among them, low dose thiazide-diuretics are the first line of drug treatment for most hypertensive patient without complications (Hill and Smith, 2005).

In selecting the classes of drugs for treatment, circadian rhythms have to be taken into consideration whenever possible due to the efficacies of many antihypertensive drugs vary at different times of administration (Lemmer, 2006). For instance, it is advisable that antihypertensive drugs given to dippers (nightly drop in BP) should be in the early morning,

whereas in non-dippers it should be at evening; or add an extra evening dose in addition to morning dose (Lemmer, 2006).

Hypertension becomes more difficult to be treated if resistant hypertension develops. Resistant hypertension means that BP of the patient is consistently above the goal level despite being treated with a combination therapy of at least three different classes of antihypertensive agents (Viera and Hinderliter, 2009). Non-compliance of patient is considered the main reason for resistant hypertension (Thrall *et al*, 2004) and this can be due to misunderstanding of patient about the medication regimen, adverse side-effects of drugs or poor patient-doctor relationship (Thrall *et al*, 2004). An interesting study conducted by (Ross *et al*, 2004) shows that hypertensive patients who believed that the treatment is necessary for them or who are more confident that the treatment actually works are likely to be more compliant to the said treatment. In addition to the five major classes of drugs, other types of drugs are direct vasodilators, alpha-1 adrenoceptor blocker and central alpha-2 agonist. Table 1.4 shows the examples for the classes antihypertensive drugs and their possible side-effects.



NOTES: ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; BB, beta blocker; CCB, calcium channel blocker; AldoAnt, aldosterone antagonist

Figure 1.5 : The algorithm for the treatment of hypertension according to The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (adapted from Chobanian *et al*, 2003).

Table 1.4 : Classes of antihypertensive drugs, examples and possible side-effects

Class	Drug	Possible side-effects
Diuretics	Chlorothiazide	Hypokalaemia
	Hydrochlorothiazide	Aggravation of gout
	Furosemide	Hyperglycaemia
	Torsemide	Impotence
	Amiloride	
	Triamterene	
Beta blockers	Atenolol	Aggravation of bronchial asthma
	Bisoprolol	Precipitation of cardiac failure
	Propranolol	Mask the impending hypoglycaemia symptoms of diabetic patient
	Acebutolol	
	Pendutolol	
	Pindolol	
Calcium channel blockers	Diltiazem	Flushing of the skin
	Verapamil	Headaches
	Amlodipine	Dizziness
	Felodipine	Gravitational oedema
	Nicardipine	Bradycardia
	Nisoldipine	Precipitation of cardiac failure
Angiotensin receptor blockers	Candesartan	Prolong hypovolaemia caused by diuretics
	Irbesartan	Precipitate renal failure
	Losartan	
	Olmesartan	
	telmisartan	
	Valsartan	
Angiotensin-converting enzyme inhibitors	Captopril	Prolong hypovolaemia caused by diuretics
	Enalapril	Angioedema
	Lisinopril	Dry cough
	Perindopril	
	Ramipril	
	trandolapril	
Direct vasodilators	Hydralazine	Reflex tachycardia
	Minoxidil	Fluid retention
		Induce autoimmune condition
Alpha-1 adrenergic blockers	Prazosin	Postural hypotension
	Doxazosin	Retrograde ejaculation of seminal fluid into bladder
	Terazosin	
Central alpha-2 agonist and centrally acting drugs	Clonidine	Drowsiness
	Methyldopa	Depression
	Reserpine	Salt and water retention

(Adapted from Chobanian *et al*, 2003 and Roger *et al*, 2011)

1.4.4.1 Angiotensin-converting enzyme inhibitor

An ACEI acts by inhibiting the activity of ACE to decrease the level of Ang II, thus causing vasodilation that lowers the TPR and hence the BP. Other effects of ACEI include increasing BK levels, reducing sympathetic nervous system activities, and lowering aldosterone levels. The ACEIs are popular among antihypertensive patient as well as CVD patient because in addition to the inhibition of ACE activity, they have other beneficial effects especially on the hearts and kidneys (Corti *et al*, 2001; Comini *et al*, 2007). With the exception of captopril and lisinopril, all ACEIs used in the clinics are prodrugs that need to be bioactivated by the liver and each of them has different potency, plasma half-life, efficacy and affinity for tissue ACE (Roger *et al*, 2011).

1.4.4.2 Vasopeptidase inhibitor

Two of the most important vasodilators in the body are BK and NPs. Bradykinin is mainly degraded by ACE (Turner and Hooper, 2002) whereas NPs are degraded primarily by NEP (Ruschitzka *et al*, 2001). Infusion of NPs to hypertensive patient has been proven to be able to decrease BP by increasing natriuresis, but this method is not practical because NP is orally inactive (Xu *et al*, 2004) and the production cost is high (Corti *et al*, 2001). Thus, inhibition of ACE or NEP may become a powerful target for combating hypertension. However, both ACEI and NEP inhibitor (NEPI) have their drawbacks. One of the weaknesses for ACEI is that ACEI is less responsive in some patients which may be due to when ACE is inhibited by ACEI, RAS is being activated continuously or kinins such as BK may be continuously metabolised by NEP (Fielitz *et al*, 2002; Xu *et al*, 2004). Similarly, NEPI is less efficient in reducing BP due to its nonspecificity (Sagnella, 2002) and it also increases the production of a vasoconstrictor, ET-1 (Daull *et al*, 2007). Although NEPI has

weak antihypertensive effect, it was found to be more effective than ACEI in the treatment of salt- and volume- dependent hypertensive rats (Pham *et al*, 1993).

Since ACE and NEP have the same active site, vasopeptidase inhibitor (VPI) which inhibits both enzymes, may overcome their respective disadvantages (Xu *et al*, 2004). Vasopeptidase inhibitors refer to agents that can simultaneously block at least two of the three vasopeptidases, ACE, NEP and endothelin-converting enzyme (Ruschitzka *et al*, 2001). Among the VPIs, simultaneous inhibition of both ACE and NEP is the most popular treatment form and the VPI discussed here refers to ACE/NEP inhibition. The overall effect of VPI is to inhibit the activity of vasopeptidases, decrease the production of Ang II and to potentiate the effects of NPs and BK, which leads to increased diuresis and vasodilation that produce BP lowering and cardioprotective effects (Xu *et al*, 2004). It is said to have better BP reducing effect and cardioprotective effect than either ACEI or NEPI alone, which may due to more complete protection of kinins (Xu *et al*, 2004). However, the main concern arises from VPI is the often fatal side-effect of angioedema which occurs at a higher rate than its counterparts (Sagnella, 2002). The safety issue regarding angioedema is the main reason that led to the decision of Food and Drug Administration (FDA) to disapprove the popular VPI omapatrilat (Song and White, 2001). Since then, development of potential VPIs is limited.

1.4.4.3 Combination therapy

When a single drug fails to achieve its goal, usually combination therapy will be considered. Combination therapy for hypertension involves combined use of two or more different classes of antihypertensive drugs (Gradman *et al*, 2010). Combination therapy uses the synergistic effect of each component and is considered to be more effective in lowering BP than monotherapy, and with less dose-dependent side-effects (Gradman *et al*,

2010). Hypertensive patients who are receiving combination therapy take either two or more different medications separately or take them in fixed-drug combination form (Rosenthal and Gavras, 2006). Fixed-drug combination involves fewer pills, better tolerability, lower cost, increased convenience and it is also faster to achieve the BP reduction target but the main disadvantages are loss of flexibility and unclear causes of side-effects (Rosenthal and Gavras, 2006). Examples of combination therapies that are available in market include diuretic + BB, ACEI + diuretic, ACEI + CCB and CCB + BB (Rosenthal and Gavras, 2006).

1.4.4.4 Vaccine for hypertension

Since the success rate for managing hypertension is low and the main regulatory mechanism of BP is the RAS, an active immunisation strategy against Ang II has been mooted and it is now in phase II clinical trial (Ambuhl *et al*, 2007). Immunisation has obvious benefits in that it does not require daily dosing because of the longer-lasting effect. This vaccine, CYT006-AngQb (AngQb), is a conjugate vaccine which uses Ang II linked to a recombinant virus-like particle (Ambuhl *et al*, 2007). Several studies have shown that this vaccine is safe with no severe side-effect, has 100% immune response with half-life about 4 months after the third booster dose, is reversible and the BP lowering effect is similar with that of low-dose direct renin inhibitor (Ambuhl *et al*, 2007; Tissot *et al*, 2008).

1.5 Complementary and alternative medicine

1.5.1 Overview

Complementary and alternative medicine (CAM) represents groups of medical practice that fall outside of conventional therapies, and their efficacies and safeties may not have been scientifically proven (Mainardi *et al*, 2009). Examples of CAM that are being commonly practised are yoga, meditation, acupuncture, herbal and supplementary medicine, massage, chiropractic and spiritual healing (Miller *et al*, 2004). In the United States of America, there appears to be a tendency for patients to seek CAM practitioners for treatment than to look for conventional physicians (Frishman *et al*, 2009). In addition, according to the World Health Organisation (WHO), 80% of the world population use plant-derived medicine (Gurib-Fakim, 2006) and among the traditional medicine from various countries, traditional Chinese medicine (TCM) seems to be growing rapidly (Davidson *et al*, 2003). The TCM is used either as an adjunct or an alternative to Western medicine (Davidson *et al*, 2003).

Among CAM, herbal and supplemental therapy is growing at the fastest speed (Buck and Michel, 2000). In fact many of the commercially available drugs are derived from herbs (Frishman *et al*, 2009) such as ephedrine from *Ephedra sinica* (Ma Huang) (Lee, 2011), digitoxin from *Digitalis purpurea* (foxglove) (Warren, 1986), salicin (source of aspirin) from *Salix alba* (willow bark) (Meier *et al*, 1988) and reserpine from *Rauwolfia serpentine* (snakeroot) (Cieri, 1998). The routes of administration of herbs include oral, nasal, topical, rectal, bathe and subcutaneous or intramuscular injection (Gurib-Fakim, 2006). However, several problems are encountered especially when using herbs that are without the approval of FDA including drug safety and standardisation of preparation (Valli and Giardina, 2002).

1.5.2 Herb-drug interactions

Herbs, although being natural, are not without side-effects after consuming. There are always misconceptions that herbs are safe without side-effects and herbs, are panacea and the efficacies of herbs can be obtained over a wide-range of doses (Chang, 2000). Indeed, concurrent use of medicinal herbs with drugs may produce the same effect, magnify or even counteract the effect of drugs and drugs that are effective at one dose can become toxic at another dose (Fugh-Berman, 2000). Many reports regarding the side-effects of concurrent use of herbs with drugs are well documented which include increased risk of bleeding between anticoagulants (e.g. warfarin) and garlic, ginkgo, ginseng or dong quai (Evans, 2000); increased BP between ACEIs and St John's wort (Buck and Michel, 2000); decreased antidepressant effect between antidepressants and yohimbine (Kearney *et al*, 2010); increased risk of toxicity between BBs, decongestants and Ma Huang (White *et al*, 1997) and also increased sedation between central nervous system depressants and valerian (Buck and Michel, 2000).

Extensive studies on herbs of different pharmacological effects are being explored and carried out. These comprise of antidiabetic, anticancer, antiulcer, lipids lowering as well as the antihypertensive effects. Examples of medicinal herbs that are scientifically shown to be effective in treating hypertension are *Rauwolfia serpentina*, *Stephania tetrandra*, *Lingusticum wallichii* and *Uncaria rhynchophylla* (Frishman *et al*, 2009). Different modes of antihypertensive actions have also been found from medicinal herbs and some of the findings are listed in Table 1.5.

Table 1.5 : Examples of antihypertensive medicinal herbs with different types of mechanisms of action.

Modes of action	Medicinal plants (Common name)	Parts of plants	Solvents	References
Diuretics	<i>Orthosiphon stamineus</i> (Cat's whiskers)	Leaves	Aqueous	(Adam <i>et al</i> , 2009)
	<i>Coriandrum sativum</i> (Yuen sai)	Seeds	Aqueous	(Aissaoui <i>et al</i> , 2008)
	<i>Bidens odorata</i> (Mozote blanco)	Aerial parts	Aqueous	(Camargo <i>et al</i> , 2004)
	<i>Tropaeolum majus</i> (Chaguinha)	Leaves	Hydroethanol	(Gasparotto <i>et al</i> , 2009)
	<i>Spilanthes acmella</i> (Akkirakaran)	Flowers	Aqueous	(Ratnasooriya <i>et al</i> , 2004)
	<i>Lepidium sativum</i> (Hab arachad)	Seeds	Aqueous	(Maghrani <i>et al</i> , 2005)
Calcium channel blockers	<i>Laelia autumnalis</i> (Orquidea)	Flowers	Methanol	(Vergara-Galicia <i>et al</i> , 2008)
	<i>Laelia anceps</i>	Roots	Methanol	(Vergara-Galicia <i>et al</i> , 2010)
	<i>Valeriana officinalis</i> (Valerian)	Roots	Ethanol, aqueous	(Circosta <i>et al</i> , 2007)
	<i>Hibiscus sabdariffa</i> (Sour tea)	Calyces	Methanol	(Ajay <i>et al</i> , 2007)
Angiotensin-converting enzyme inhibitors	<i>Clerodendron trichotomum</i>	Stems	Ethyl acetate	(Kang <i>et al</i> , 2003)
	<i>Rabdosia coetsa</i>	Whole plant	Ethyl acetate	(Li <i>et al</i> , 2008)
	<i>Cuscuta japonica</i>	Seeds	Ethyl acetate	(Oh <i>et al</i> , 2002)
	<i>Tribulus terrestris</i>	Fruits	Aqueous	(Sharifi <i>et al</i> , 2003)
	<i>Musanga cecropioides</i> (Umbrella tree)	Stems	Aqueous	(Adeneye <i>et al</i> , 2006)
Angiotensin receptor blockers	<i>Salvia elegans</i> (Mirto)	Aerial parts	Hydroethanol	(Jimenez-Ferrer <i>et al</i> , 2010)
	<i>Bocconia frutescens</i>	Roots	Methanol/ dichloromethane	(Caballero-George <i>et al</i> , 2003)
	<i>Citrus limetta</i>	Leaves	Aqueous	(Perez <i>et al</i> , 2010)
	<i>Astragalus complanatus</i>	Seeds	Total flavonoid fraction in distilled water	(Xue <i>et al</i> , 2008)
	<i>Hippophae rhamnoides</i> (Shaji)	Seeds	Total flavones in distilled water	(Pang <i>et al</i> , 2008)

‘Table 1.5, continued’

Potassium channel activation	<i>Brillantaisia nitens</i>	Leaves	Methylene chloride/methanol	(Dimo <i>et al</i> , 2007)
	<i>Valeriana wallichii</i> (Indian valerian)	Rhizomes	Chloroform, aqueous	(Gilani <i>et al</i> , 2005)
	<i>Ulmus macrocarpa</i>	Roots	Ethanol	(Oh <i>et al</i> , 2008)
Sympatholegic activities	<i>Jacaranda minosaefolia</i> (Jacaranda)	Leaves	Hydromethanol	(Nicasio and Meckes, 2005)
	<i>Eugenia uniflora</i> (Pitanga)	Leaves	Aqueous	(Consolini <i>et al</i> , 1999)
	<i>Rhodiola sacra</i> (Plateau ginseng)	Roots	Aqueous	(Shih <i>et al</i> , 2008)
Nitric oxide and endothelium dependant vasodilation	<i>Mansoa hirsute</i>	Leaves	Ethanol	(Campana <i>et al</i> , 2009)
	<i>Maytenus ilicifolia</i>	Leaves	Ethanol	(Crestani <i>et al</i> , 2009)
	<i>Fritillaria ussuriensis</i> (Beimu)	Bulbs	Aqueous	(Kang <i>et al</i> , 2002)
	<i>Geum japonicum</i>	Whole plant	Butanol	(Xie <i>et al</i> , 2007)

1.6 Methods and techniques

1.6.1 Fractionation, purification and isolation of natural products

Natural products, especially herbs, have been sources of therapeutic agents since ancient times (Spainhour, 2005). Recently, in a period between 1981 to 2002, it was found that among the 74 synthetic drugs approved for the treatment of hypertension, 48 of them were found to be of plant origin (Newman *et al*, 2003).

The methods of consumption that traditionally practised such as chewing the raw plants or boiling the plants have several weaknesses such as variation in the amount of active compounds, reaction among other compounds that coexist with the bioactive compounds and change or loss of bioactivity due to variation in collection, processing and storage of the plant (Colegate and Molyneux, 2008). Scientific ways to obtain bioactive compounds from natural products are needed and involve detection and screening, isolation

and purification, and structure identification of bioactive compounds (Spainhour, 2005; Colegate and Molyneux, 2008).

Detection and screening of potential bioactive plants are done by various *in vitro* as well as *in vivo* assays. After identifying certain bioactive plants, the next step is isolation and purification which involves various techniques and technologies. The first step is usually extraction of the plant by using solvents with different polarities. The next step involves further solvent partition and extensive chromatography methods by considering acidity, polarity and molecular size of the compounds (Colegate and Molyneux, 2008). These involve techniques such as solid-phase extraction, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), counter current chromatography, desalting and ion exchange chromatography (Spainhour, 2005). Today, the last step structural elucidation of bioactive compounds uses advanced technologies and techniques such as nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), ultraviolet (UV)/visible and infrared absorption spectroscopy and also 'hyphenated' technologies such as HPLC-MS, HPLC-NMR and MS-MS (Spainhour, 2005; Colegate and Molyneux, 2008).

1.6.2 Blood pressure measurement methods

Measurement of BP in laboratory rats can be divided into direct and indirect methods. Both of these methods have their advantages and disadvantages and the choice of method depends on the objectives of the study (Kurtz *et al*, 2005).

In indirect method, it is non-invasive which means no surgical procedure is required. The most common indirect method is the use of a cuff in which changes in the blood flow in a tail or limb is detected by the occlusion or release of the cuff (Kurtz *et al*, 2005). The advantages of this method are: (1) non-invasive; (2) less expensive compared to other method such as telemetry; and (3) can be used to study changes in BP in large groups of animals over a short or long period of time (Van Vliet *et al*, 2000; Kurtz *et al*, 2005). In contrast, the main disadvantage is that this method requires physical restraint and heating that poses stress to the animal (Irvine *et al*, 1997). This method is thus advised to be used in study in which large BP difference is expected (Gross and Luft, 2003).

In direct method, it is invasive and involves surgical procedure. A sensor device is implanted into the arterial system in rat to measure the arterial BP. There are three common types of sensing devices: fluid-filled catheters, transducer-tipped catheters and radiotelemetries (Van Vliet *et al*, 2000). Fluid-filled catheter is the oldest and the most widely used direct method. It uses a heparin-filled catheter to be inserted into a major artery of rat while the other end of the catheter is connected to a BP transducer. This method can be used in acute as well as chronic measurement of BP in animals. The main advantage of this fluid-filled catheter method is that it produces accurate and reliable readings and permits infusion of various experimental agents (Kurtz *et al*, 2005). However, this method imposes some stress to animals because the animals are not free-moving and the presence of clotting around the catheter tips can affect the BP readings (Kurtz *et al*, 2005). Moreover, there is also a risk of infection because it involves surgical procedure.

Although transducer-tipped catheter is more sensitive, it is more expensive and only suitable for short term study (Van Vliet *et al*, 2000). The method of radiotelemetry can be used in wider areas of studies. Radiotelemetry uses a BP sensor (radiotelemetric probe) which is implanted in the animal and adopts an electrical device to receive and process the signal. The advantages include: (1) measures BP of free moving conscious animals; (2) causes less stress as no restraint of animal is required; (3) less labor intensive; (4) more efficient, convenience and reliable; (5) measures BP for a longer duration compared to the catheterised method; (6) no anesthesia is involved during BP measurement; (7) can measure BP around the clock (Mills *et al*, 2000; Van Vliet *et al*, 2000). The main problem arises from radiotelemetry is its high cost, requires more technical skills and also has the risk of infection (Kurtz *et al*, 2005).

1.6.3 *In vitro* vascular tension studies

To study the pharmacological effect of vasoactive agents on smooth muscles and their drug-receptor interaction, strips or ring segments of arteries isolated from laboratory animals such as rats and rabbits are used (Furchgott and Bhadrakom, 1953). In this *in vitro* method, the strips are removed from laboratory animals and mounted with stainless steel S-hooks in physiological solutions (eg. Krebs-bicarbonate solution) bubbled with 95% oxygen and 5% carbon dioxide in organ chambers (Furchgott and Bhadrakom, 1953). The strips or ring segments of arteries are attached to force transducer to record the changes in tension in arteries to various vasoactive drugs (Gonzales *et al*, 2000). One of the most popular findings using this *in vitro* vascular tension method was the discovery of endothelial-derived hyperpolarising factor which involves the release of NO that causes the relaxation of VSM (Furchgott, 1999). Earlier studies using this method have shown that epinephrine, norepinephrine and ACh caused vasocontraction whereas sodium nitrite

caused vasorelaxation (Furchgott and Bhadrakom, 1953), but later studies proved that ACh is a vasorelaxant agent rather than a vasoconstrictor agent (Furchgott, 1999).

1.6.4 Laboratory animals

Spontaneously hypertensive (SHR) rat is the most widely used animal model for hypertension. It belongs to the phenotype-driven genetic model of hypertension (Lerman *et al*, 2005). The SHR strain was obtained by selective breeding of spontaneously hypertensive Wistar-Kyoto (WKY) male rat with relatively higher BP female rat (Okamoto and Aoki, 1963). This strain of rat is widely used in hypertension studies because the incidence of hypertension is 100%, it is sensitive to various antihypertensive agents which are also effective in human (Yamori and Okamoto, 1973) and it presents hypertensive complications (Yamori and Okamoto, 1973). Other than that, stroke prone SHR and arteriolipidosis-prone rats were developed from SHR as a model for stroke and atherosclerosis (Yamori, 1978).

The most commonly used control strain for SHR rat is WKY rat (Johnson *et al*, 1992). Initially it was thought that all WKY rats constituted a single inbred strain. Later studies showed that there was a great biological variability among WKY rats themselves and also between SHR and WKY rats (Kurtz *et al*, 1989; Johnson *et al*, 1992). Although these findings were discovered, WKY rat is still extensively used as a control for SHR because SHR was derived from WKY rat (Kurtz and Morris, 1987).

Another strain of rats that are widely studied and extensively used in various research fields are Sprague-Dawley (SD) rats. This strain was produced by the Sprague-Dawley Company, Madison, Wisconsin, in 1925. The main advantages of SD rat are its docile character that makes it easier to be handled and also its excellent reproductive performance.

1.6.5 Nuclear magnetic resonance spectroscopy and mass spectrometer

One of the most powerful instruments to obtain the structure of an organic compound is NMR spectroscopy. This instrument uses a principle in which when a compound containing nuclei such as ^1H or ^{13}C is put in a strong magnetic field and simultaneously irradiated with certain electromagnetic energy; the absorption of energy is quantized by the NMR spectroscopy and displayed as NMR spectra which can be either 1 dimensional (D) or 2D as well. The most common nuclei used in NMR spectroscopy are ^1H and ^{13}C .

Nuclear magnetic resonance spectroscopy usually works together with MS for a better understanding of the molecule. The MS provides the molecular weight (MW) of a certain molecule whereas NMR spectroscopy tells the connectivity between atoms in a molecule. In comparison, NMR spectroscopy has an advantage over MS that it is not destructive (the sample can be recovered) although more amount of sample is needed for NMR spectroscopy.

1.6.6 *In vitro* assay of angiotensin-converting enzyme activity

Inhibition of ACE has been an effective therapeutic approach for hypertension and ACE inhibitory compounds can be found in some plants. There are researches carrying out to search for potential ACE inhibitory compounds from plants (Hansen *et al*, 1995; Duncan *et al*, 1999; Lacaille *et al*, 2001). Many methods have been used to study the potential ACE inhibitory activities from compounds isolated from plants or crude extracts from plants. These methods use spectrophotometry, fluorometry, HPLC or radiochemistry (Lam le *et al*, 2007).

In 1991, a new method, based on the cleavage of chromophore- and fluorophore-labeled substrate, dansyltriglycine by ACE into dansylglycine, which was measured by HPLC, was introduced (Elbl and Wagner, 1991). From the past times until now, many methods, either modified or with new principles are introduced and adopted in many studies. For example, in 2005, a cost-effective and high throughput method with the absorbance of final product measured by using microtitre plate reader was introduced (Serra *et al*, 2005). Then, a method which used a new substrate known as 3-hydroxybutyrylglycyl-glycyl-glycine, was claimed to be more sensitive, rapid and accurate than conventional method (Lam le *et al*, 2007). Later that, a rapid, sensitive, high-throughput and advanced colorimetric assay to measure hippuric acid (HA) has also been created (Jimsheena and Gowda, 2009).

Although there are plenty of methods to measure ACE activity, the colorimetric assay introduced by Cushman and Cheong by using synthetic peptide, hippuryl-L-histidyl-L-leucine (HHL) as a substrate is the most popular (Jimsheena and Gowda, 2009). In this method, HHL is hydrolysed by ACE to form HA and histidyl-leucine (HL). The amount of HA produced is proportional to the ACE activity which are then extracted and measured by spectrophotometer at 228 nm (Cushman and Cheung, 1971). This method has some

drawbacks: it is time-consuming, unhydrolysed HHL may be extracted and this method is not suitable for high-throughput screening (Jimsheena and Gowda, 2009).

Since the method by Cushman and Cheung, 1971 has several disadvantages; a method which based on colorimetric determination of HA with reduced interference has been developed (Hurst and Lovell-Smith, 1981). In this method, no extraction of HA is required because the HA produced reacts directly with the colorimetric reagent, cyanuric chloride (2,4,6-trichloro-*s*-triazine) in dioxane. This reaction produces a coloured complex that can be quantified from its absorbance at 382 nm (Hayakari *et al*, 1978). The principle for this method is shown in Figure 1.6 (Hoe *et al*, 2007).

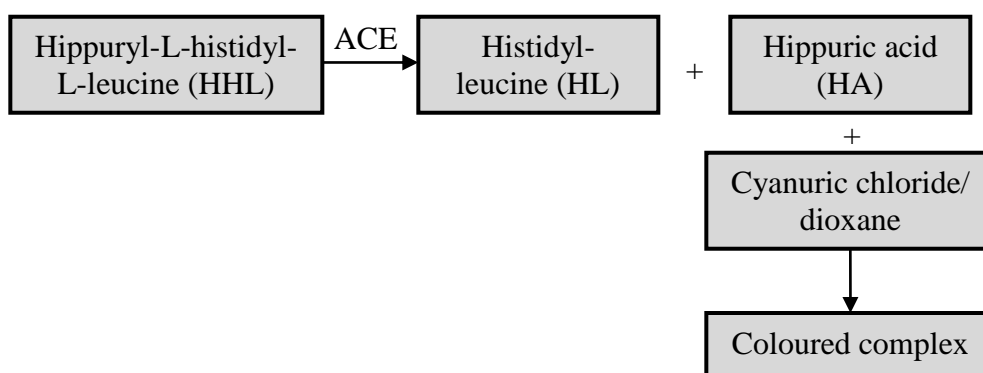


Figure 1.6 : The principle for the measurement of angiotensin-converting enzyme activity by a colorimetric assay (adapted from Hurst and Lovell-Smith, 1981).

1.6.7 *In vitro* assay of neutral endopeptidase activity

In search of potential ACE inhibitory compounds from plants, NEP inhibitory compounds, although less popular, can be found in some plants as well (Kiss *et al*, 2005). Methods with the principles of radiochemistry, colorimetric, enzyme-linked immunosorbent assay and the most popular fluorometric method have been used to study the NEP activities in tissues or sera (Yandle *et al*, 1992; Tudoric *et al*, 2000). The synthetic substrates that are being used include glutaryl-Ala-Ala-Phe-amidomethylcoumarin(Yandle *et al*, 1992), N-dansyl-Ala-Gly-D-nitro-Phe-Gly (DAGNPG) (Jiang *et al*, 2004) and Suc-L-Ala-L-Ala-Phe-7-amino-4-methylcoumarin (SAAP-AMC) (Kiss *et al*, 2005).

Until now, methods to measure the NEP activities have been modified and transferred to screen for potential NEP inhibitory compounds from plants. For example, a two-step assay based on Bormann and Melzig (Bormann and Melzig, 2000) has been widely used by Kiss A (Kiss *et al*, 2005; Kiss *et al*, 2006; Kiss *et al*, 2008). In this two-step assay, the first reaction is initiated by the addition of NEP to the substrate SAAP-AMC to form Phe-7-amino-4-methylcoumarin (P-AMC). The second reaction is the action of leucine aminopeptidase (APN) on P-AMC to form 7-amino-4-methylcoumarin (AMC) which is fluorescence and measured by spectrofluorometer at $\lambda_{\text{excitation}} = 355 \text{ nm}$ and $\lambda_{\text{emission}} = 460 \text{ nm}$. The two-step reaction is shown in Figure 1.7.

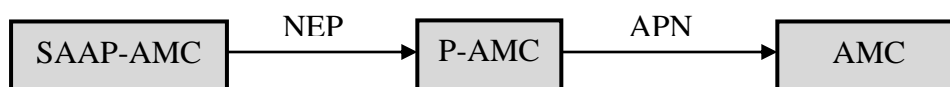


Figure 1.7 : The two-step reaction that finally produces fluorescence 7-amino-4-methylcoumarin (adapted from Bormann and Melzig, 2000).

1.6.8 Effects of proteases on plant extract

Proteases are enzymes that hydrolyse proteins and they are involved in many physiological reactions in the human body. One of the reactions is the digestion process wherein some of the important proteases are pepsin, trypsin and chymotrypsin (Guyton and Hall², 2006). These enzymes may change the actions or functions of the compounds that taken into the body.

Study by Hoe *et al*, 2007 postulated that the putative hypotensive agents in this plant extract seem to be peptidal substances in nature. This aspect is further studied by using NMR spectroscopy and HPLC-MS and also by the effects of these three proteases on ACE inhibitory activities of the plant extract. Loss of ACE inhibitory activity of plant extract may show that the extract is degraded by proteases and it is most probably a kind of protein.

1.7 Plant material

1.7.1 Family Asteraceae/ Compositae

Family Asteraceae or Compositae comprises of herbs or shrubs, but are rarely trees or vines (Mbagwu *et al*, 2009). Their leaves are simple, opposite or alternate but rarely compound (Mbagwu *et al*, 2009). There are involucre to support the flower heads and the flowers have ray florets at the margin and disc florets at the central (Mbagwu *et al*, 2009). Most members of Asteraceae or Compositae undergo asexual reproduction by seed (Noyes, 2007). Family Asteraceae comprises of 1,600 genera and 23,000 species is the largest family of flowering plants (Gao *et al*, 2010). This family is widely distributed on all continents except Antarctica.

Asteraceae has a wide range of uses such as in medicine, ornament and economy as well (Gao *et al*, 2010). In medical application, *Artemisia annua* which contains antimalarial compound, artemisinin, has been listed in the Chinese Pharmacopoeia to treat fevers such as malaria (Mueller *et al*, 2000). It is also found that *Saussurea involucre*, listed in the Chinese Pharmacopoeia, has anticancer, anti-inflammation and free radical scavenging activities (Wu *et al*, 2009). *Gynura procumbens* (*G. procumbens*), which will be discussed later is also popular among traditional medicine.

1.7.2 *Gynura procumbens*

Table 1.6 : Taxonomy of *Gynura procumbens*.

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Asterales
Family	Asteraceae or Compositae
Genus	<i>Gynura</i>
Species	<i>procumbens</i>

(adapted from Botanica Sistemática, 2009)

Gynura procumbens (Lour.) Merr. is a weak climbing or scrambling perennial herb. In Malaysia, its common names are akar sebiak, sambung nyawa, daun dewa, kelemai merah, acham akar and dewa raja. Its stems can grow up to 6 meters long, with smooth or sparsely pubescent. Young stems are green and become light purple with green spots when mature. The leaves are almost oval-shaped with size of 3-10cm X 0.5-3cm. The lower surfaces of the leaves are purplish. *Gynura procumbens* can be found in Central and Western Africa, Southeast Asia, China and Papua New Guinea. This plant is fast growing and easy to breed by using stem cuttings (Globinmed, 2010). Figure 1.8 shows the photo of *G. procumbens* planted in Kuala Kangsar, Perak, Malaysia.

In folk medicine, the plant is used for treating kidney diseases, fever and rashes (Perry, 1980) and for hypertension. Scientifically, *G. procumbens* is found to have the following properties:

1. Antihypertensive (Lam *et al*, 1998; Hoe and Lam, 2005)
2. Anti-ulcer (Mahmood *et al*, 2010)
3. Anti-oxidant (Puangpronpitag *et al*, 2010)
4. Antidiabetic and antihyperlipidemic (Zhang and Tan², 2000)



Figure 1.8 : *Gynura procumbens* (Lour.) Merr.

1.8 Research objectives

Hypertension is one of the major health problems worldwide and most of the conventional treatments have side-effects. Searching of potential antihypertensive plants has been an alternative in belief that the plant, being natural may have lesser side-effects. Previous studies from our laboratory have shown that a partially purified fraction (FA-I) from final aqueous fraction (FA), from *G. procumbens* possesses antihypertensive effect by inhibiting ACE (Hoe *et al*, 2007) and blocking calcium channels (Hoe *et al*, 2011). The present study was designed with the following objectives:

1. To explore the effects and mechanism of FA-I on Ang II-induced contraction of aortic rings as well as hypotensive and vasodilatory effects of BK
2. To test the dual ACE/NEP inhibitory activities of FA-I and its subfractions
3. To study the acute oral antihypertensive effects of FA-I
4. To characterise bioactive FA-I subfractions

CHAPTER 2 - MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant material

Whole plants of *G. procumbens* were collected from Peninsular Malaysia. Three voucher specimens (KLU 047690, KLU 047691 and KLU 047692) have been deposited in the Herbarium at Rimba Ilmu, University of Malaya. The photo below shows one of the voucher specimens with herbarium number KLU 047690.



2.1.2 Animals

Adult male SD, SHR and WKY rats, weighing between 250-320 g, were obtained from the Experimental Animal Care unit, University of Malaya and kept under standard conditions. Normal commercial rat chow (Gold Coin Animal Feed, Malaysia) and tap water were provided *ad libitum*. All studies on experimental animals have been conducted under protocols reviewed and approved by the Animal Ethics Committee, University of Malaya Medical Center (Ethics Reference Number: FIS 14/ 10/ 2009/ 0112/ PTF (R)).

2.1.3 Chemicals and reagents

All the chemicals and reagents used were of analytical grade unless otherwise stated: Ang I (AnaSpec, INC, USA), heparin sodium 5,000 I.U. (LEO Pharmaceutical Products Ltd., Ballerup, Denmark), SephadexTM LH-20 (Amersham Bioscience AB, Uppsala, Sweden), sodium hydroxide (NaOH) (Fisher Scientific, U.K.) and Nembutal sodium pentobarbitone (CEVA Sante Animale, Libourne, France).

Chemicals and reagents from Merck KGaA, Darmstadt, Germany are Ang II, BK, boric acid (H_3BO_3), calcium chloride (CaCl_2), glucose, indomethacin, magnesium sulphate (MgSO_4), NEP from porcine kidney, ninhydrin, phosphoramidon, potassium dihydrogen phosphate (KH_2PO_4), potassium chloride (KCl), silica gel 60 F₂₅₄ TLC aluminium sheets, sodium bicarbonate (NaHCO_3), sodium chloride (NaCl) and 1,4-dioxan, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES).

Apart from that, ACh chloride, APN, bovine chymotrypsin, bovine trypsin, cyanuric chloride, HA, HHL acetate, hydrochloric acid (HCl), N^ω-nitro-L-arginine methyl ester (L-NAME), pepsin from porcine gastric mucous membrane, phenylephrine (PE) hydrochloride, rabbit ACE and SAAP-AMC were produced by Sigma-Aldrich Co., St Louis, MO, USA. The solvents acetic acid, acetone, ethanol, ethyl acetate, hexane, methanol and *n*-butanol are from R & M Marketing, Essex, U.K.

Rat chows were purchase from Gold Coin Feedmills (M) Sdn Bhd, Selangor, Malaysia.

2.1.4 Equipment

The main equipments used in this study were:

Vacuum evaporation system consisting of a rotary evaporator, an oil bath (model OSB-2000) and an aspirator (model A-1000S) from Eyela Tokyo Rikakikai Co., Ltd., Japan, with a low temperature circulator (model WCR-P8) from Daihan Scientific Co., Ltd., Seoul, Korea.

Digital physiographic recording system (PowerLab, ADInstruments Pty. Ltd., Australia) consisting of a PowerLab Data Acquisition System (model ML866), a BridgeAmp (model ML224), a BioAmp (model ML136), a NIBP controller (model ML125), a force transducer (model MLT050/D) and a BP transducer (model MLT0380/D) that is controlled by a HP personal computer (model HPL1908w) loaded with the PowerLab Chart Version 5.0 Software.

Other equipments include a chromatography column (2.5 cm, i.d. x 75 cm, height), fraction collector (Bio-Rad 2100, Bio-Rad Laboratories, U.S.A.), reagent spray (Camag, Switzerland), viewing cabinet (Chromato-vue CC-10, U.K.) combined with multi-band 254/365nm lamp (UVGL-58), micro tube pump (Eyela MP-3, Japan), NMR spectrometer 400 MHz (JEOL, JEOL Ltd, Japan), microprocessor-based bench-top pH meter (Hanna[®] pH 211, Hanna[®] Instruments, U.S.A.), centrifuge (Jouan CR 312, American Laboratory Trading, Inc, U.S.A.), UV-VIS spectrophotometer (Model UV mini 1240, Shimadzu, Germany), spectrofluorometer (Model Fluoroskan Ascent FL, Thermo Scientific, U.K.).

2.2 Extraction and fractionation of *Gynura procumbens* leaves

The protocols for extraction and fractionation follow the method used by Hoe, 2007 (Figure 2.1). Briefly, leaves from fresh *G. procumbens* were cleaned and dried in an oven at 40°C and then ground to powder. They were then processed through the following steps in a sequential manner ie extract in 96% ethanol and 80% aqueous ethanol, defatted in hexane and fractionated by water-saturated butanol to obtain the final aqueous fraction (FA), which was then passed through a column containing Sephadex LH-20 gel (Appendix A) to obtain FA-I fraction (Figure 2.1). The FA-I fraction was subjected to preparative TLC (Appendix B) resulted in several FA-I subfractions (FA-Ia – FA-Ie) that were revealed by ninhydrin reagent (Appendix C). Using a bioassay guided protocol, the FA-I subfractions that were tested for positive bioactivities were subsequently sent for characterisation by ^1H NMR 400 MHz spectrometer (JEOL, Japan). The FA-Ia subfraction was analysed more extensively by ^{13}C NMR 100 MHz, 2D distortionless enhancement by polarisation transfer (DEPT) NMR 100 MHz, infrared spectroscopy and LCMS.

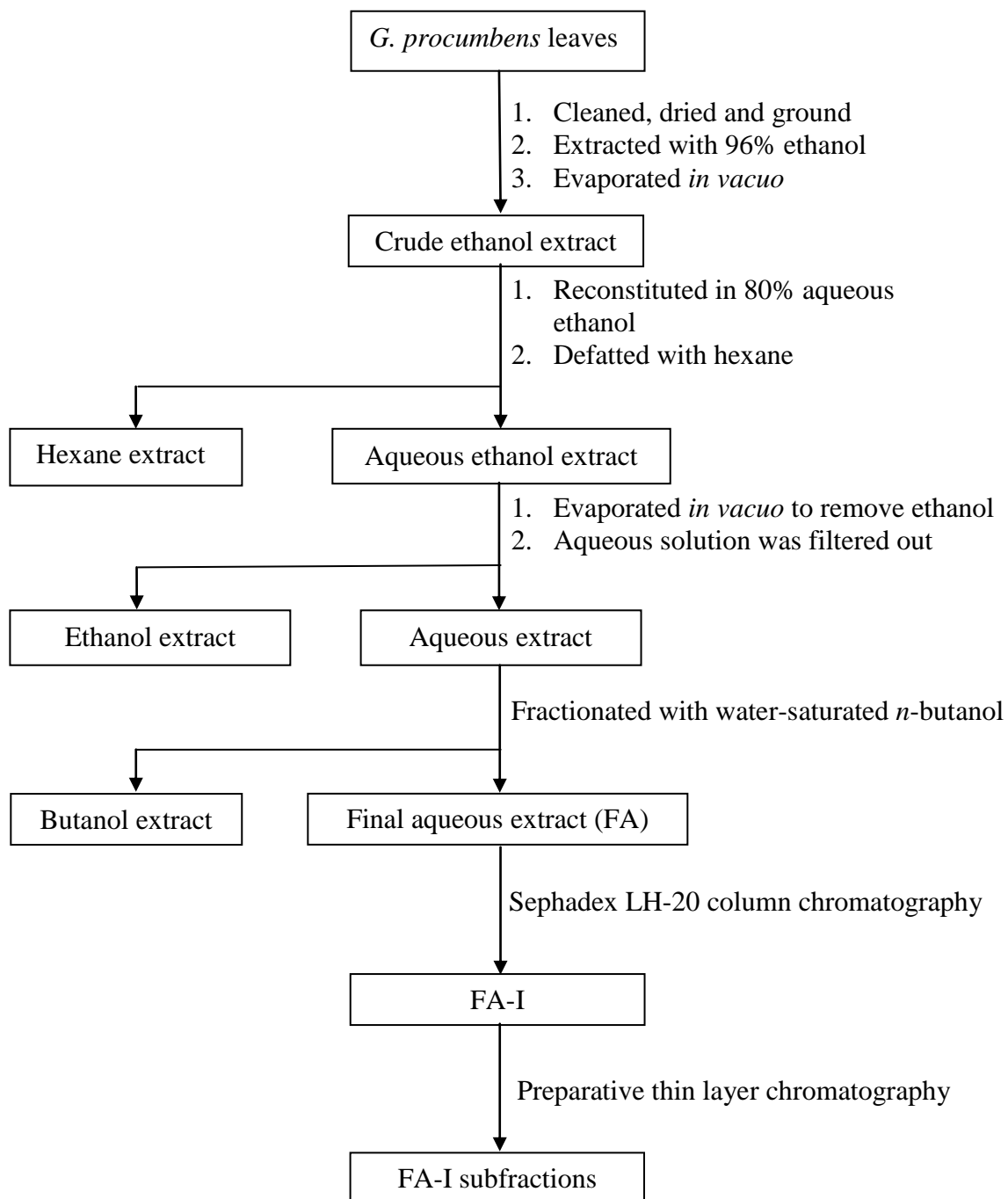


Figure 2.1 : Extraction and fractionation of *Gynura procumbens* that finally produce the FA-I subfractions.

2.3 *In vitro* vascular tension studies

The effects of FA-I on blood vessels were studied using *in vitro* techniques on isolated rat thoracic aorta.

2.3.1 Preparation of rat aortic rings

The descending thoracic aorta was removed from male SD rats and mounted in ice-cold oxygenated Krebs-Henseleit (K-H) solution (Appendix D). The isometric contractile force of the aorta was recorded by the PowerLab computer system. The procedures for preparation of rat aortic rings are presented below while Figure 2.2 shows a photograph of an aortic ring in an organ bath chamber.

For some preparations, the endothelial layer of aortic ring was mechanically removed by inserting a pair of fine forceps into the lumen and gently rotating the aorta around the forceps. These endothelium-denuded rings were similarly equilibrated in organ bath for 60 min. The success of the endothelial denudation was tested by precontracting the rings with 1.0×10^{-6} M PE and observing the response to 1.0×10^{-5} M ACh. A lack of any relaxation of the PE-precontracted rings indicated that the endothelium was satisfactorily removed whilst relaxation of at least 70% of the PE-induced contraction showed that the endothelial layer was still present. The instructions for the preparation of aortic rings are shown below:

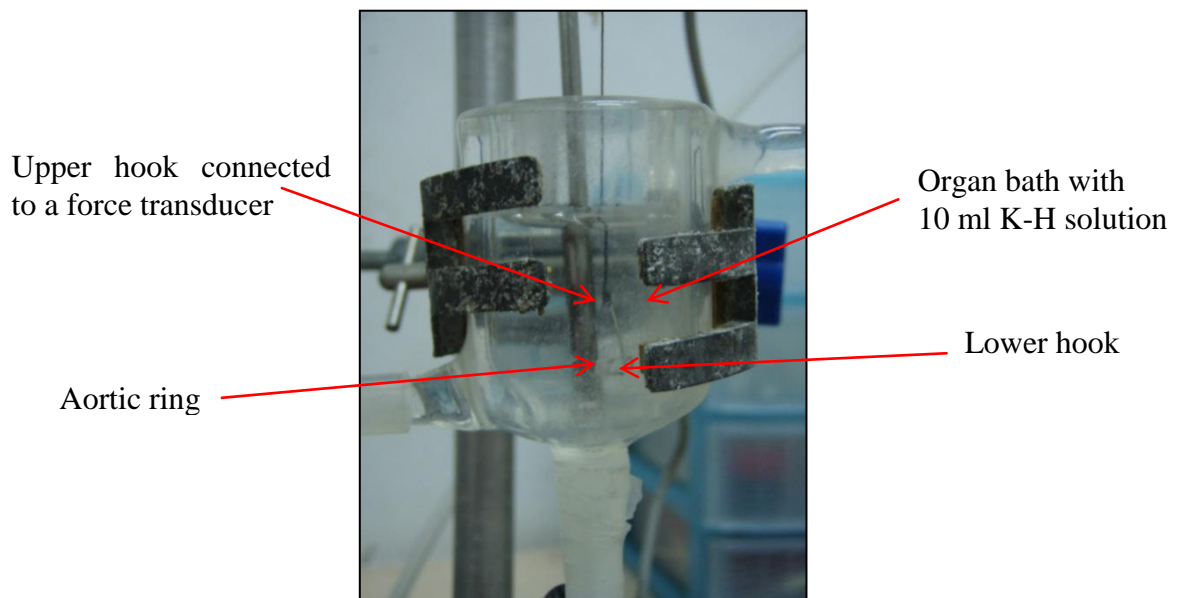
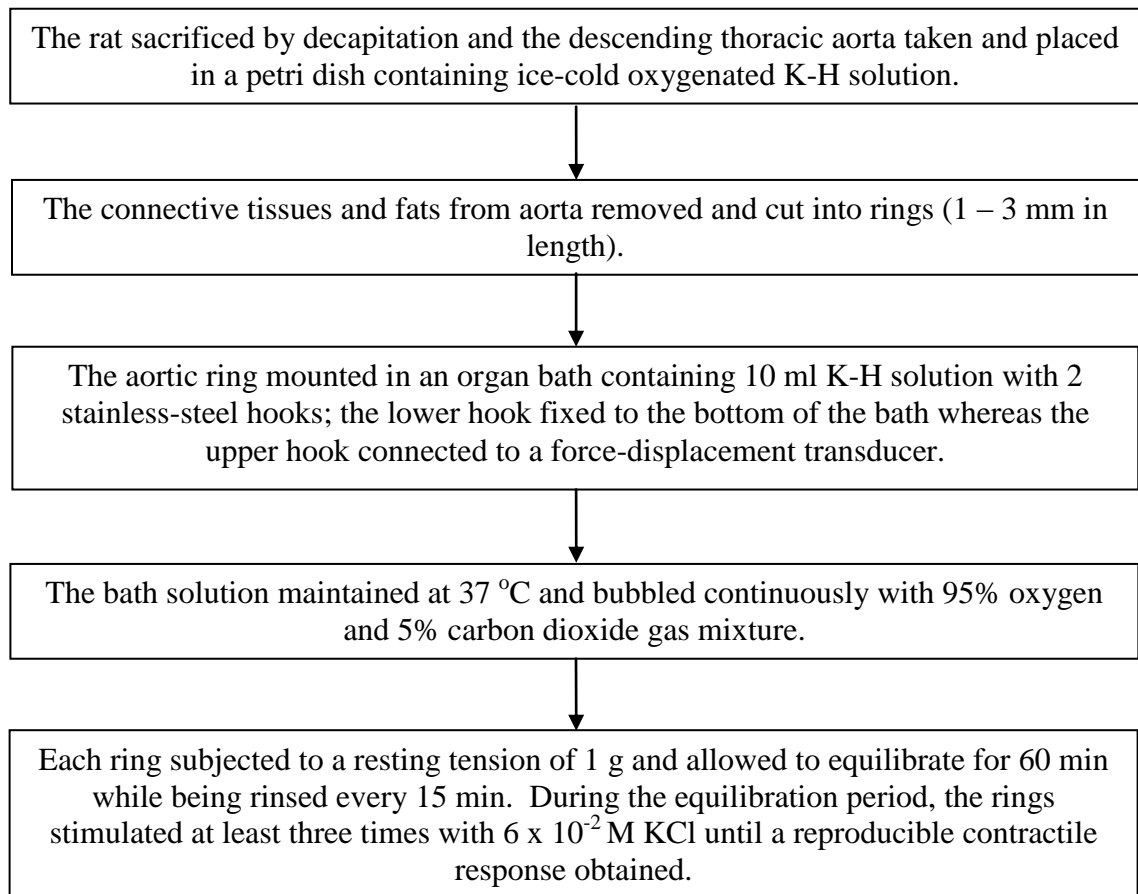


Figure 2.2 : The aortic ring was mounted in an organ bath containing 10 ml Krebs-Henseleit solution with the lower hook being fixed to the bottom of the bath whereas the upper hook is connected to a force-displacement transducer connected to a digital physiographic setup.

2.3.2 Effects of FA-I pretreatment on angiotensin I-induced contraction

Endothelium-intact (n = 6) and -denuded aortic rings (n = 6) were preincubated for 20 min in the absence or presence of FA-I (final concentration: 1.0×10^{-4} and 1.0×10^{-3} g/ml) (with reference to Hoe, 2007). After the incubation period, contraction of the rings was evoked by adding cumulative concentrations of Ang I ($1 \times 10^{-8.5}$ M - 1×10^{-6} M) and their responses in the absence or presence of FA-I were recorded.

2.3.3 Effects of FA-I pretreatment on angiotensin II-induced contraction

Endothelium-intact (n = 6) and -denuded aortic rings (n = 6) were preincubated for 20 min in the absence or presence of FA-I (final concentration: 1.0×10^{-4} and 1.0×10^{-3} g/ml). After the incubation period, contraction of the rings was evoked by adding cumulative concentrations of Ang II ($1 \times 10^{-8.5}$ M – 1×10^{-6} M) and their responses in the absence or presence of FA-I were recorded.

2.3.4 Effects of FA-I pretreatment on angiotensin II-induced contraction of aortic rings in the presence of N^ω-nitro-L-arginine methyl ester or indomethacin

To study the involvement of endothelium in the response to Ang II brought by FA-I, endothelium-intact rings (n = 6) were preincubated for 20 min with or without FA-I (final concentration: 1.0×10^{-4} and 1.0×10^{-3} g/ml) and indomethacin (final concentration: 10 μ M), a non-selective COX inhibitor or L-NAME (final concentration: 0.1 μ M), an eNOS inhibitor, and followed by the cumulative additions of Ang II.

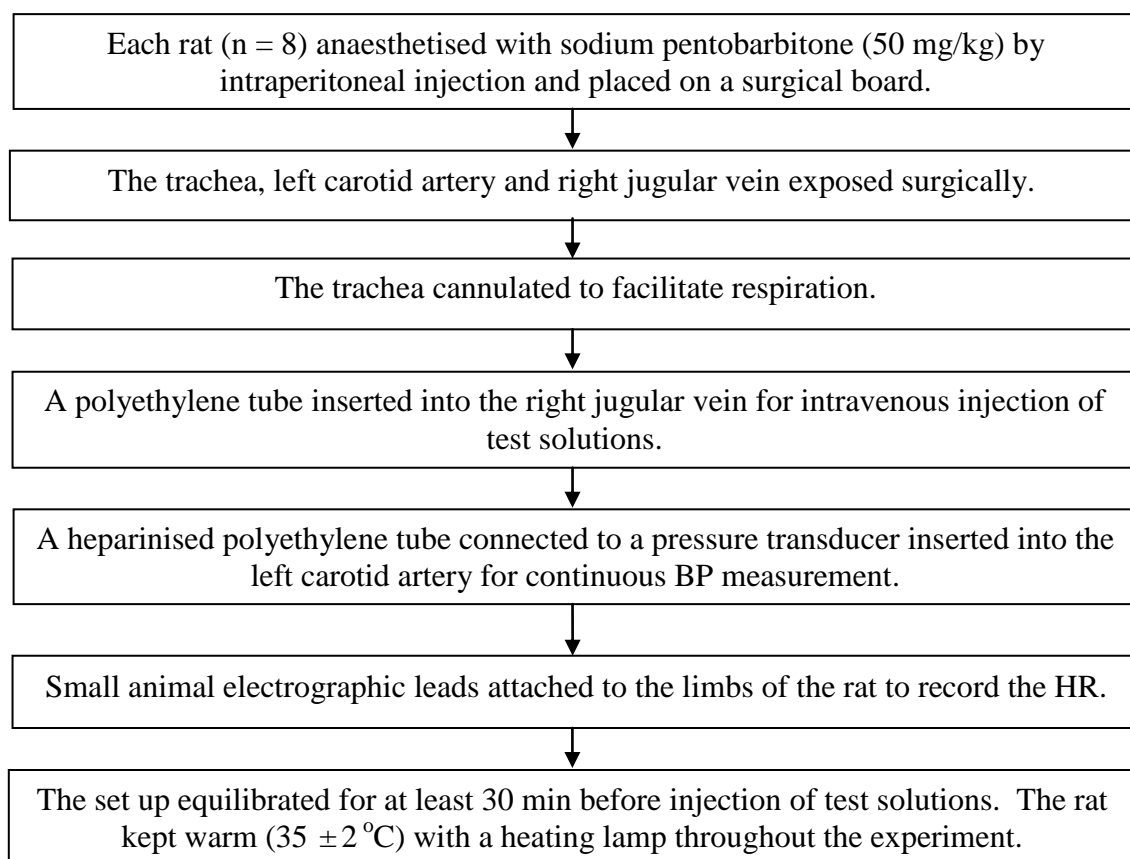
2.3.5 Effects of FA-I pretreatment on bradykinin-induced relaxation of phenylephrine-precontracted aortic rings

After the equilibration period, intact aortic rings (n = 8) were precontracted again with PE (1.0×10^{-6} M). The contractions of aortic rings to PE were allowed to reach a

plateau before the cumulative addition of BK ($1.0 \times 10^{-11} \text{ M} - 1.0 \times 10^{-6} \text{ M}$). The relaxation responses of PE-precontracted aortic rings to the cumulative additions of BK were determined in the presence or absence of pretreatment with FA-I (final concentration: 1.0×10^{-4} and $1.0 \times 10^{-3} \text{ g/ml}$) 20 min before the sequential additions of PE and BK.

2.4 Effects of FA-I on the action of bradykinin on mean arterial pressure and heart rate of rats

The BP was recorded by a pressure transducer connected to a PowerLab setup. The procedures for direct BP measurement in rat are shown as follows:



In this method, heparinised saline (10 U/ml) was used to prevent blood coagulation and the FA-I was dissolved in normal saline. After the equilibration period, increasing doses of BK (0 – 625 ng/kg) were injected into the rat to be followed by a single

administration of FA-I (10 mg/kg) (with reference to Hoe, 2007) and then the same increasing doses of BK again. A minimum interval of 3 min was allowed between each injection of BK whilst a minimum period of at least 10 min was given between the administrations of BK and FA-I to allow the BP to return to resting state. Each injection was set at a fixed volume of 0.1 ml per 300 g rat body weight. Changes in the BP due to the administration of BK and FA-I were obtained by calculating the difference between the BP before and the lowest BP after the injections. The mean arterial pressure (MAP) was calculated using the formula: $MAP = DBP + 1/3(SBP - DBP)$. Figure 2.3 shows the photography of the set up for the measurement of BP and HR of rat.

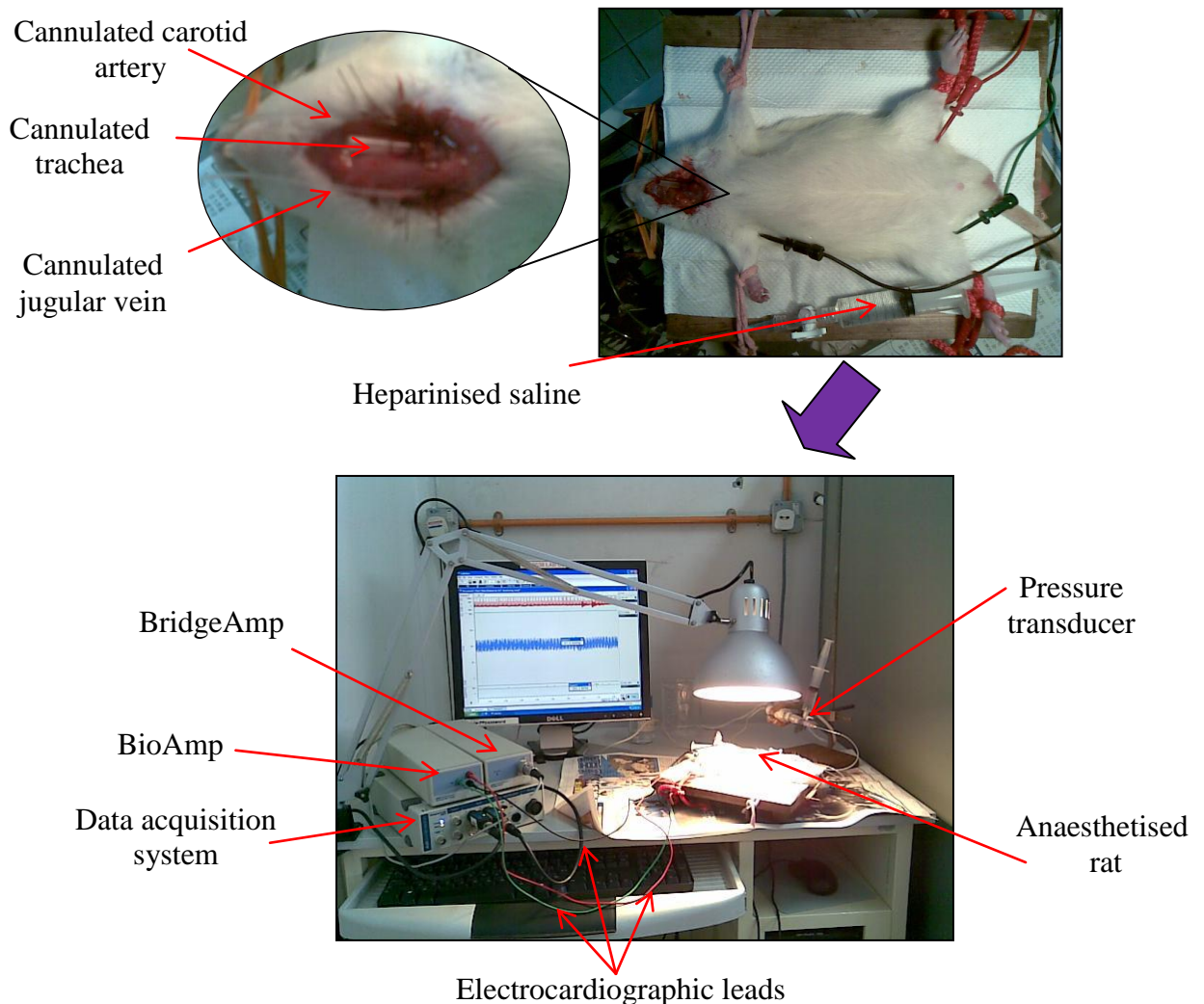
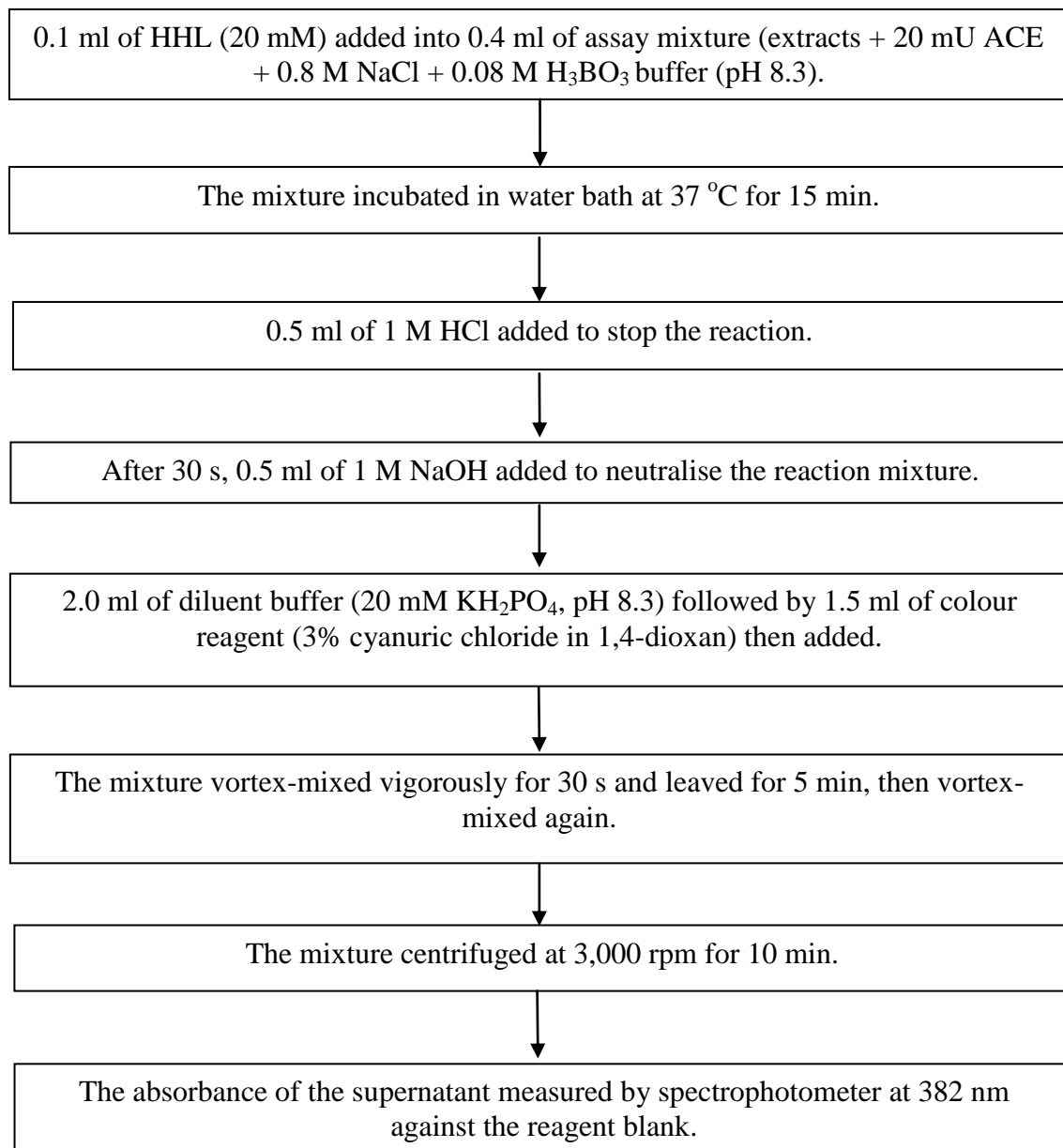


Figure 2.3 : Setup of the experimental procedure for the measurement of blood pressure and heart rate in a rat.

2.5 *In vitro* assays

2.5.1 *In vitro* effect of FA-I and its subfractions on angiotensin-converting enzyme activity

The ACE inhibitory activity of *G. procumbens* was studied by using an *in vitro* ACE colourimetric assay of Hurst and Lovell-Smith, 1981 with some modifications. The procedures for the *in vitro* ACE inhibitory activity assay are shown as follows:



In order to prepare the enzyme blank, the terminating and neutralising solutions were added before the HHL; to prepare the reagent blank, the HHL solution was replaced with distilled water whereas for hippuric acid standards preparation, the HHL solution was replaced with standard HA solutions.

For calculations, one unit of ACE activity is defined as the amount of enzyme required to produce one μ mole of HA per minute at 37 °C. The effect of FA-I and its subfractions on ACE activity was calculated by the following formula:

$$\frac{B}{A} \times 100\%$$

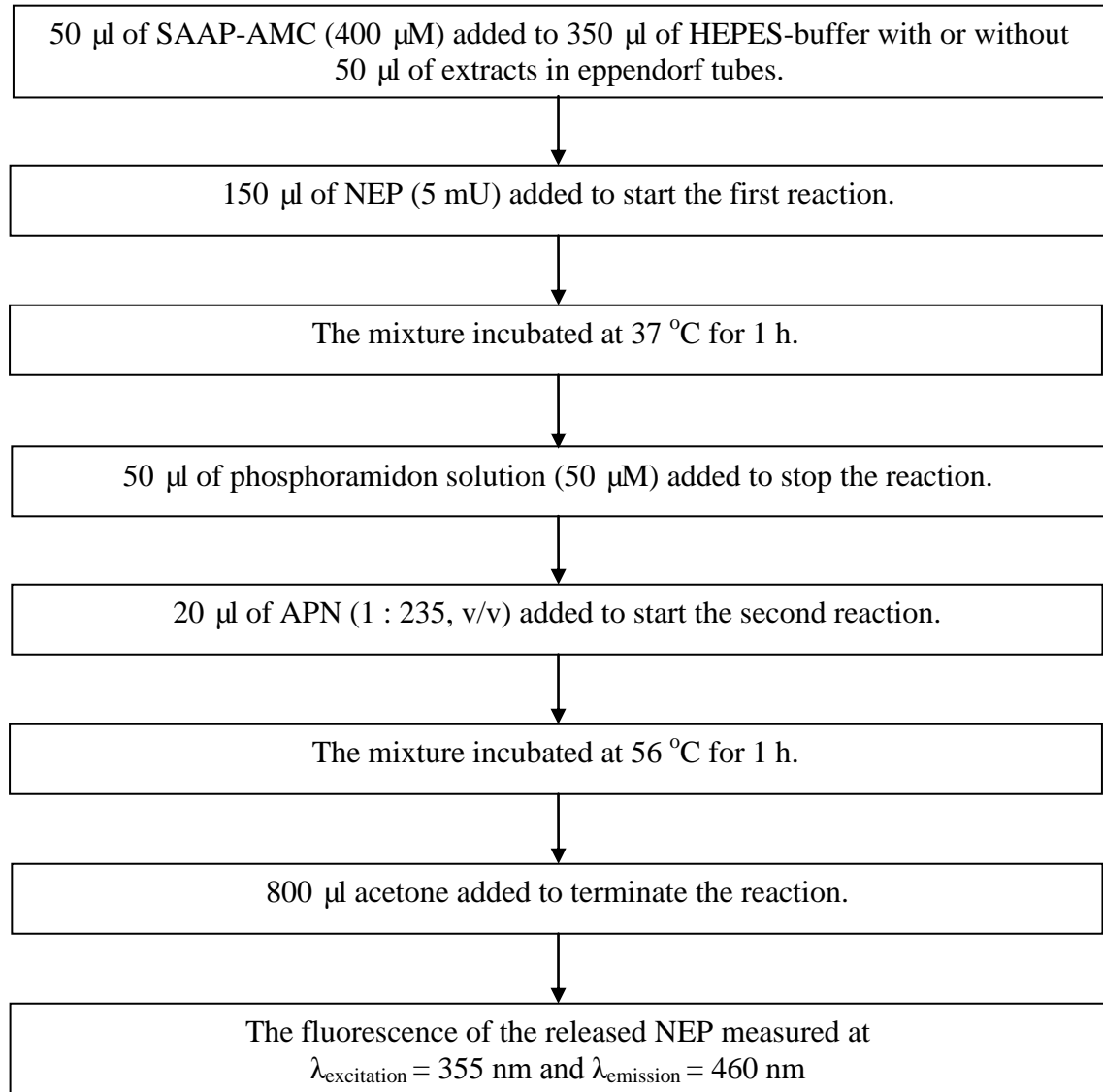
Where: A = ACE activity in the absence of inhibitor

B = ACE activity in the presence of inhibitor (plant extract)

The IC₅₀ value, which is the concentration of the fraction required to inhibit 50% of the ACE activity, was determined using linear regression analysis.

2.5.2 *In vitro* effect of FA-I and its subfractions on neutral endopeptidase activity

A two-steps assay widely used by Kiss *et al.*, 2008, with some modifications was used in this study, as shown in the following:



The effect of FA-I and its subfractions on NEP activity was calculated by the following formula:

$$I_{NEP} = [(I_{total} - I_{APN}) / (100 - I_{APN})] \times 100$$

Where: $I_{total} = 100 - (F_1 / K_0) \times 100$

$$I_{APN} = 100 - (F_2 / K_0) \times 100$$

All the measurements are fluorescent and represented as P_1 , P_2 , K_0 and K :

$$F_1 = P_1 - K \text{ and } F_2 = P_2 - K$$

Where: P_1 = Fluorescence of sample with extract

P_2 = Fluorescence of sample with extract added in second reaction
(effect of extract on APN activity)

K_0 = Fluorescence of sample without extract

K = Fluorescence of sample with extract without enzyme

2.6 Effect of orally administered FA-I on blood pressure of spontaneously hypertensive and Wistar-Kyoto rats

In this study, SHR (n = 7) and WKY (n = 8) rats were used. In at least 7 days prior to the experiment, the rats were trained to enter the restraint cage and their SBPs were measured. On the experimental day, FA-I was dissolved in distilled water and administered orally to rats at doses of 50, 100 and 200 mg/kg. Control rats were administered with same amount of distilled water. The SBP was measured by tail-cuff method with a device connected to a PowerLab setup before and also 2, 4, 6, 8, 24 h after administration. Figure 2.4 shows the setup for a rat kept in a restraint cage and its SBP was measured by a cuff connected to a PowerLab setup.

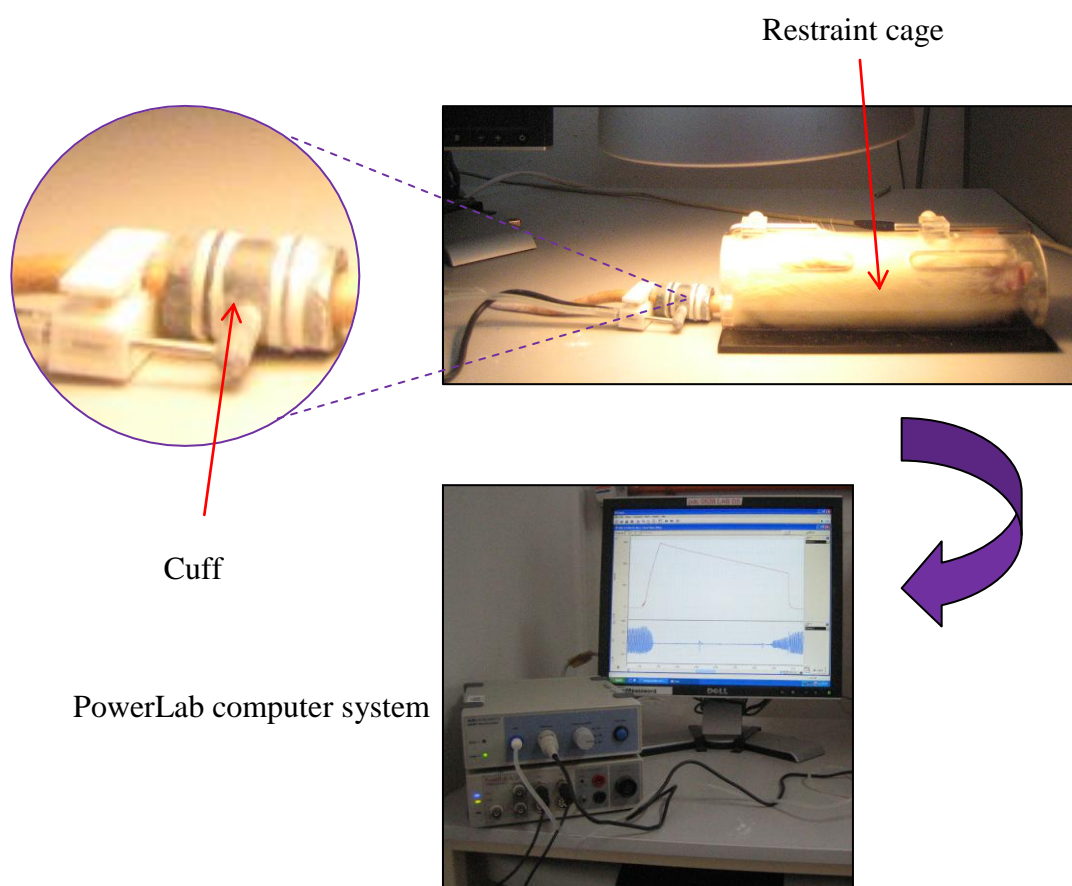


Figure 2.4 : Measurement of rat systolic blood pressure of rat by tail-cuff method.

2.7 Effect of proteases on angiotensin-converting enzyme inhibitory activity of FA-I

The stability of FA-I to maintain the ACE inhibitory activity against proteases pepsin, trypsin and chymotrypsin was assessed individually by the method of Kuba *et al*, 2003 with slight modifications. In this *in vitro* assay, FA-I (0.25 – 2 mg/ml) was mixed with 0.2 ml of 0.05% (w/v) pepsin, chymotrypsin or trypsin solution. Pepsin was dissolved in 0.1 M HCl at pH 2.0 whereas chymotrypsin and trypsin were both dissolved in 0.1 M potassium phosphate buffer at pH 8.0. The mixture was incubated at 37 °C for 6 h, after which it was boiled for 10 min to stop the digestion. Each sample was then centrifuged at 3,000 rpm for 20 min and the supernatant was collected and freeze-dried before it was sent for the measurement of ACE inhibitory activity (refer section 2.5.1).

2.8 Data analyses

When the cumulative concentration-response curves of Ang I or Ang II were examined, the contractions in the presence of FA-I were calculated as a percentage of the highest contractile response obtained in the absence of FA-I. This contractile response evoked by the highest concentration of Ang I or Ang II was designated as E_{\max} . The pEC_{50} value ($-\log EC_{50}$), determined from the EC_{50} value (the concentration required to produce a half-effect in the concentration-response curves) was calculated from individual log concentration-response curves of the Ang I or Ang II by sigmoidal non-linear regression analysis within the 95% confidence intervals using GraphPad Prism[®] v.4.00 (GraphPad Software Inc., La Jolla, CA, USA). The vasorelaxant response to BK was measured from the plateau of the PE-induced contraction and expressed as a percentage (%) relaxation of the PE-induced contraction. The IC_{50} value, which is the concentration of extracts to inhibit 50% of ACE or NEP activity, was obtained from dose-response curves by linear regression.

2.9 Statistical analyses

All values are expressed as mean \pm SEM for n number of rats or separate experiments. Statistical differences for *in vitro* studies were evaluated by one-way analysis of variance (ANOVA) while student's t -test was used for *in vivo* studies. A probability level of less than 0.05 ($p < 0.05$) was considered to be significantly different.

CHAPTER 3 - RESULTS

3.1 Extraction and fractionation of *Gynura procumbens* leaves

Gynura procumbens leaves were ground to powder form and extracted sequentially with several solvents to obtain FA-I. The FA-I fraction on TLC plates sprayed with ninhydrin reagent was shown in Figure 3.1. The yield of each solvent extract is shown in Figure 3.2. Heating on FA-I at TLC plates sprayed with ninhydrin reagent revealed five different fractions (FA-Ia – FA-Ie) with retention factor (R_f) values of 0.21, 0.31, 0.45, 0.53 and 0.66 respectively (Figure 3.2).



Figure 3.1 : Chromatogram of a partially purified aqueous fraction (FA-I) on thin layer chromatography (TLC) plates after being sprayed with ninhydrin reagent. The dark circle reveals FA-Ia.

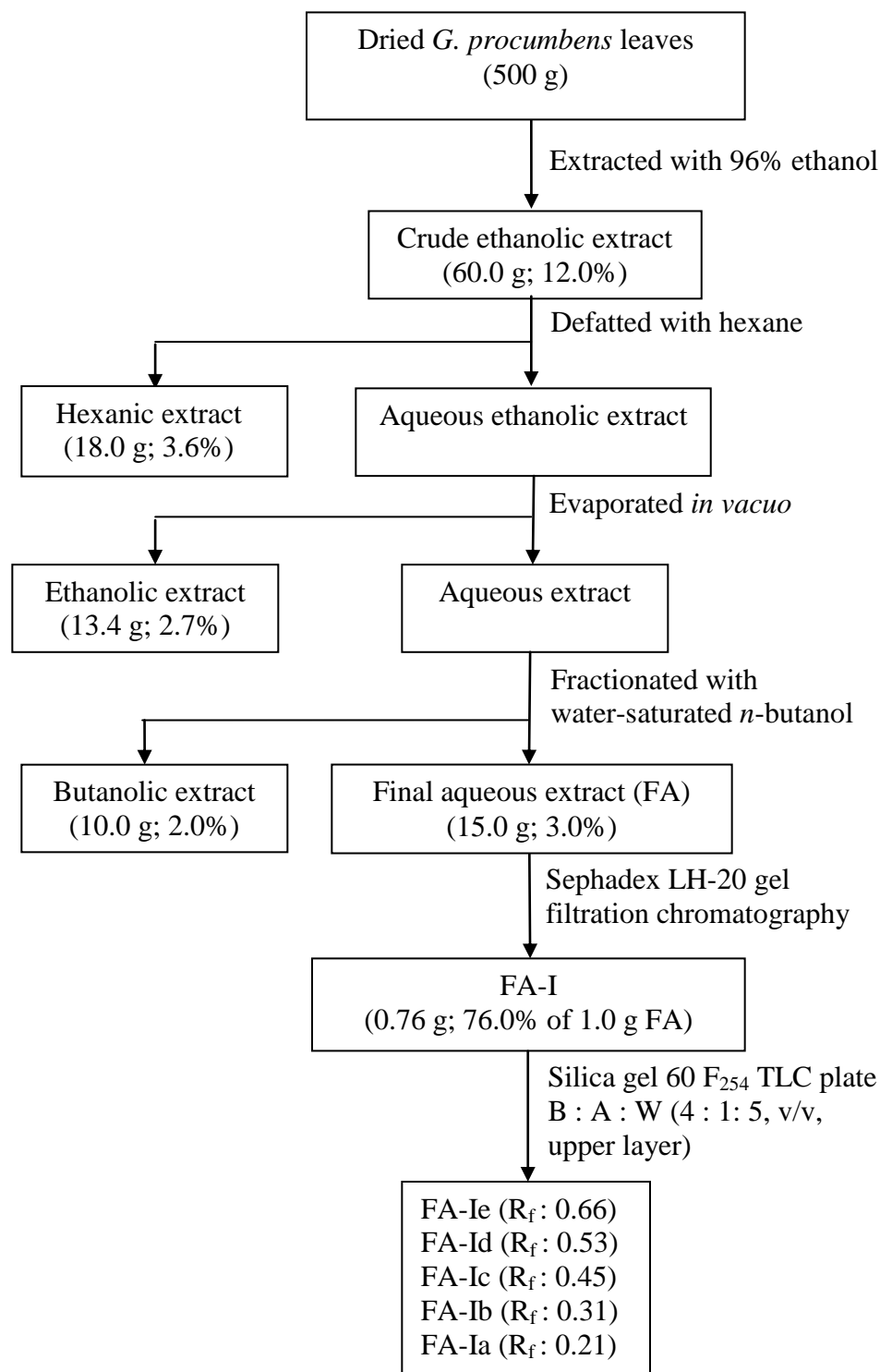


Figure 3.2 : Flow chart of extraction and fractionation of the leaves of *Gynura procumbens* (All % values in parenthesis indicate % yield of each fraction obtained from the original dried leaves weight except for FA-I which was calculated from 1 g of FA).

3.2 *In vitro* vascular tension studies

3.2.1 Effects of FA-I pretreatment on angiotensin I-induced contraction

Pretreatment with FA-I at 1.0×10^{-4} and 1.0×10^{-3} g/ml inhibited the Ang I-induced contraction of both endothelium-intact (Figure 3.3a) and -denuded (Figure 3.3b) aortic rings in a concentration-dependent manner with significant ($p < 0.01$) reduction in the E_{\max} values. Furthermore, rings that were kept intact (Figure 3.3a) relaxed significantly ($p < 0.05$) more than those that were denuded of endothelium (Figure 3.3b).

3.2.2 Effects of FA-I pretreatment on angiotensin II-induced contraction

Experiments were then carried out with endothelium-intact and -denuded aortic rings to study the involvement of endothelium in the response of FA-I to the contraction evoked by Ang II. Results show that pretreatment of endothelium-intact aortic rings with FA-I attenuated the contraction caused by Ang II (Figure 3.4a), with the E_{\max} values being significantly ($p < 0.001$) decreased when compared with controls. However, exposure of endothelium-denuded aortic rings to FA-I for 20 min before the addition of increasing concentrations of Ang II did not blunt the contractions induced by Ang II (Figure 3.4b).

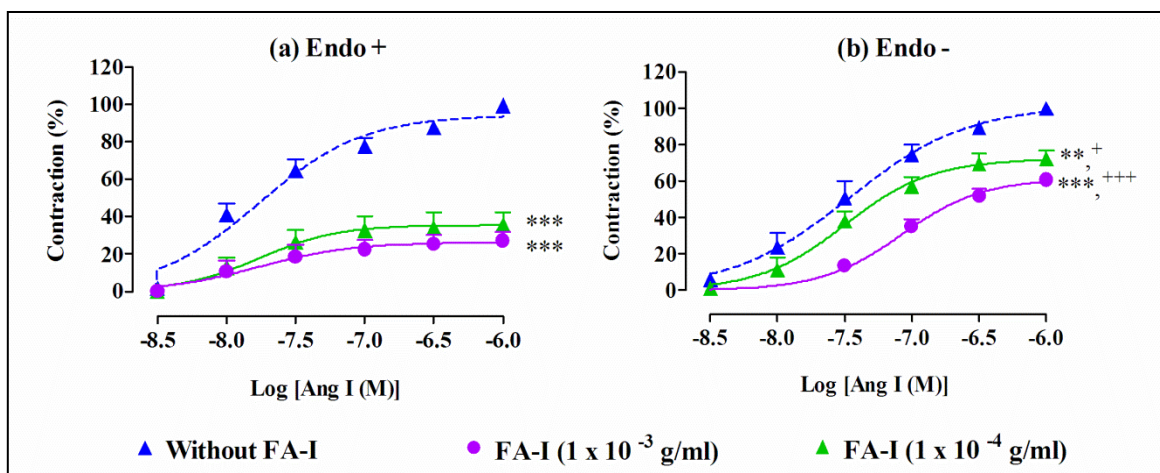


Figure 3.3 : Effects of a partially purified aqueous fraction (FA-I) on angiotensin I-induced contraction in (a) endothelium-intact (Endo +) and (b) endothelium-denuded (Endo -) aortic rings. The effects were determined in the absence or presence of FA-I (1.0×10^{-4} or 1.0×10^{-3} g/ml). Values are mean \pm S.E.M. (n = 6). **p < 0.01; ***p < 0.001 compared with controls (without FA-I) and ⁺p < 0.05; ⁺⁺⁺p < 0.001 compared with Endo +.

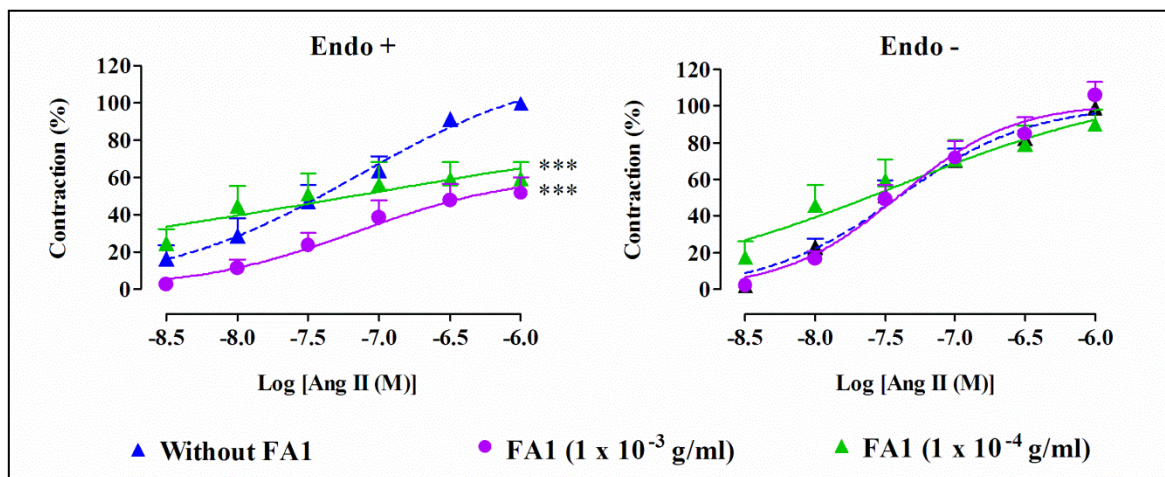


Figure 3.4 : Effect of a partially purified aqueous fraction (FA-I) on angiotensin II-induced contraction in (a) endothelium-intact (Endo +) and (b) endothelium-denuded (Endo -) aortic rings. The effect was determined in the absence (without FA-I) or presence of FA-I (1.0×10^{-4} or 1.0×10^{-3} g/ml). Values are mean \pm S.E.M. (Endo +, n = 7; Endo -, n = 6) ***p < 0.001; compared with controls (without FA-I).

3.2.3 Effects of FA-I pretreatment on Ang II-induced contraction of aortic rings in the presence of N^o-nitro-L-arginine methyl ester or indomethacin

To further study the role of endothelial factors in the action of FA-I, Ang II-induced contraction in endothelium-intact rings were performed in the presence of both FA-I and L-NAME or indomethacin. Results show that pretreatment of FA-I at 1.0×10^{-4} and 1.0×10^{-3} g/ml in the presence of L-NAME (Figure 3.5a) or indomethacin (Figure 3.5b) did not cause any significant changes in the E_{\max} values for Ang II as compared to controls.

In aortic rings pretreated with L-NAME, although there were no significant changes in E_{\max} values, the concentration-response curves of Ang II are shifted to the right (Figure 3.5a) with a significant ($p < 0.05$) decrease in pEC_{50} values (Table 3.1). Similarly, preincubation of FA-I and indomethacin also shifted the concentration-response curve to the right (Figure 3.5b), with a significant ($p < 0.01$) reduction of pEC_{50} values for Ang II in the presence of FA-I at 1.0×10^{-3} g/ml (Table 3.1). These results show that the eNOS and COX pathways may be involved in the response of FA-I on Ang II-induced contraction of aortic rings.

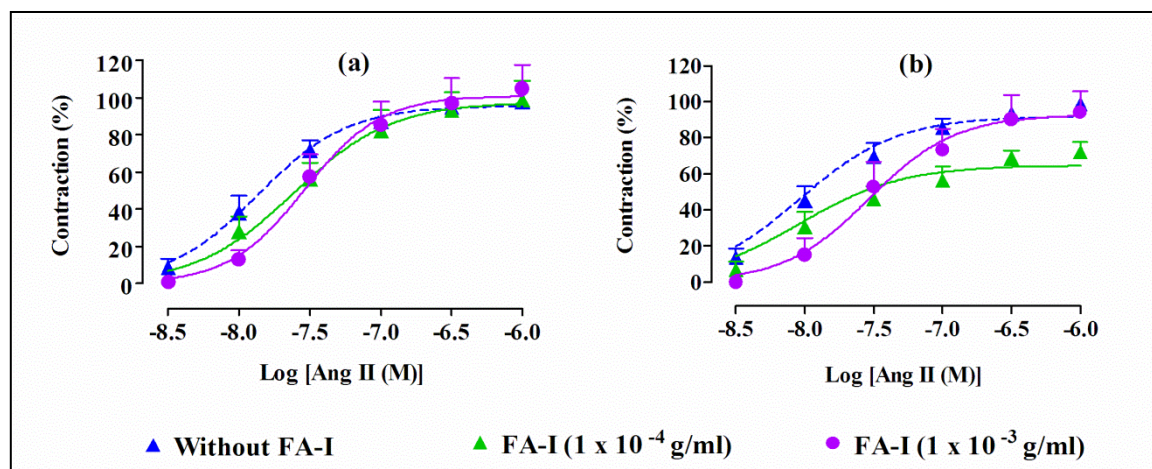


Figure 3.5 : Effect of a partially purified aqueous fraction (FA-I) on angiotensin II-induced contraction in endothelium-intact aortic rings preincubated with (a) N^o-nitro-L-arginine methyl ester (L-NAME) or (b) indomethacin. The effect was determined in the absence or presence of FA-I (1.0 x 10⁻⁴ or 1.0 x 10⁻³ g/ml). Values are mean ± S.E.M. (n = 6).

Table 3.1 : Effect of a partially purified aqueous fraction (FA-I) on the pEC₅₀ values for Ang II-induced contraction of aortic rings pretreatment with L-NAME or indomethacin.

FA-I (x 10 ⁻³ g/ml)	pEC ₅₀	
	L-NAME	Indomethacin
0	7.84 ± 0.06	7.89 ± 0.08
0.1	7.61 ± 0.12*	7.75 ± 0.16
1.0	7.54 ± 0.11**	7.53 ± 0.14**

Values are mean ± S.E.M. (n = 6)

*p < 0.05; **p < 0.01 compared with controls (without FA-I).

3.2.4 Effects of FA-I pretreatment on bradykinin-induced relaxation of phenylephrine-precontracted aortic rings

Bradykinin (1×10^{-11} – 1×10^{-6} M) dose-dependently relaxed PE-induced contraction of aortic rings. This observation was significantly ($p < 0.05$) enhanced by pretreatment of aortic rings with FA-I (Figure 3.6).

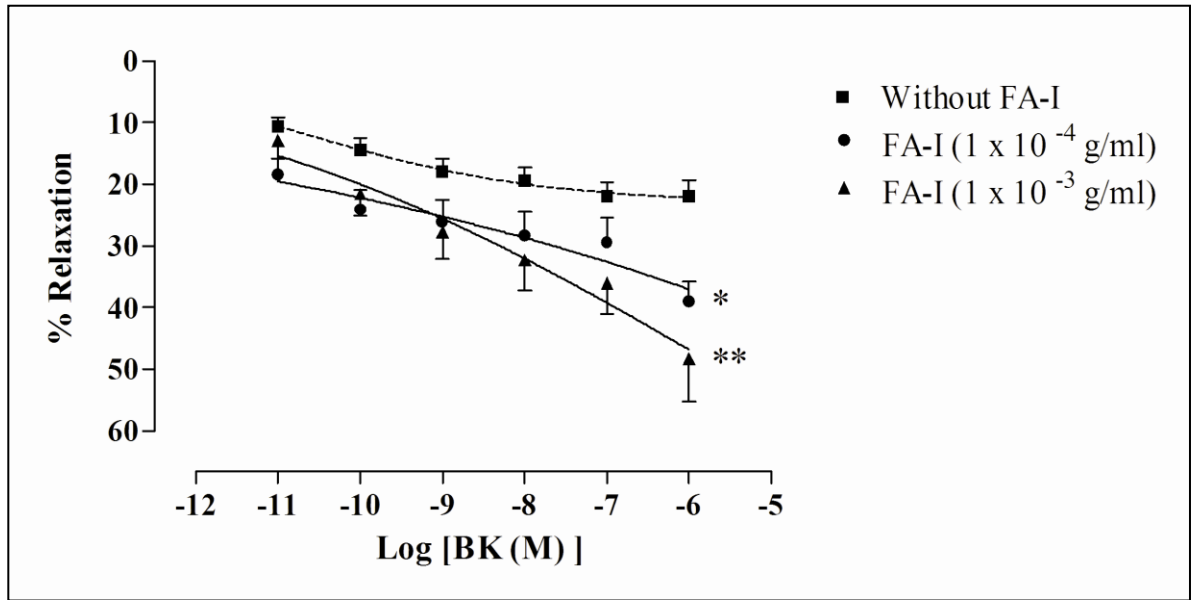


Figure 3.6 : Effect of a partially purified aqueous fraction (FA-I) on bradykinin-induced relaxation of aortic rings precontracted with phenylephrine. The effect was determined in the absence (without FA-I) or presence of FA-I (1.0×10^{-4} or 1.0×10^{-3} g/ml). Values are mean \pm S.E.M. ($n = 8$). * $p < 0.05$ ** $p < 0.01$ compared with controls (without FA-I).

3.3 Effects of FA-I on the action of bradykinin on mean arterial pressure and heart rate of rats

Intravenous administrations of BK at different doses induced immediate and significant ($p < 0.05$) decrease in the MAP (Figure 3.7a) and HR (Figure 3.7b) of rats. After pretreatment with 10 mg/kg FA-I, the BP lowering effect produced by BK was markedly ($p < 0.05$) greater. The difference in the hypotensive effect of BK before and after FA-I pretreatment became significant ($p < 0.05$) at doses of 78, 156, 312 and 625 ng/kg. Bradykinin also decreased the HR but there was no significant difference in the bradycardic effect between before and after FA-I administration. Pre-dose baseline levels were recovered within 10 min after BK administration.

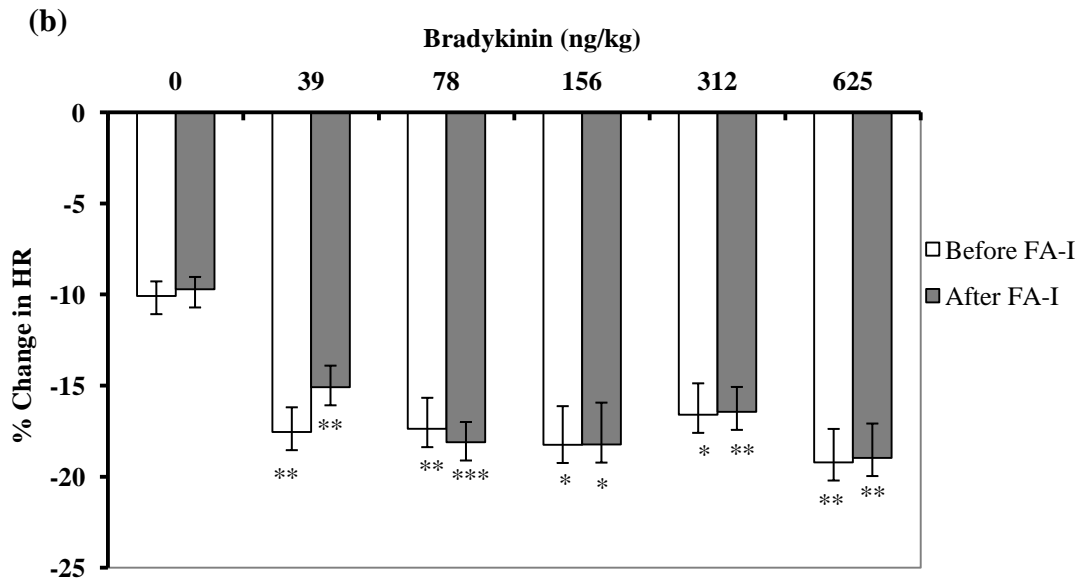
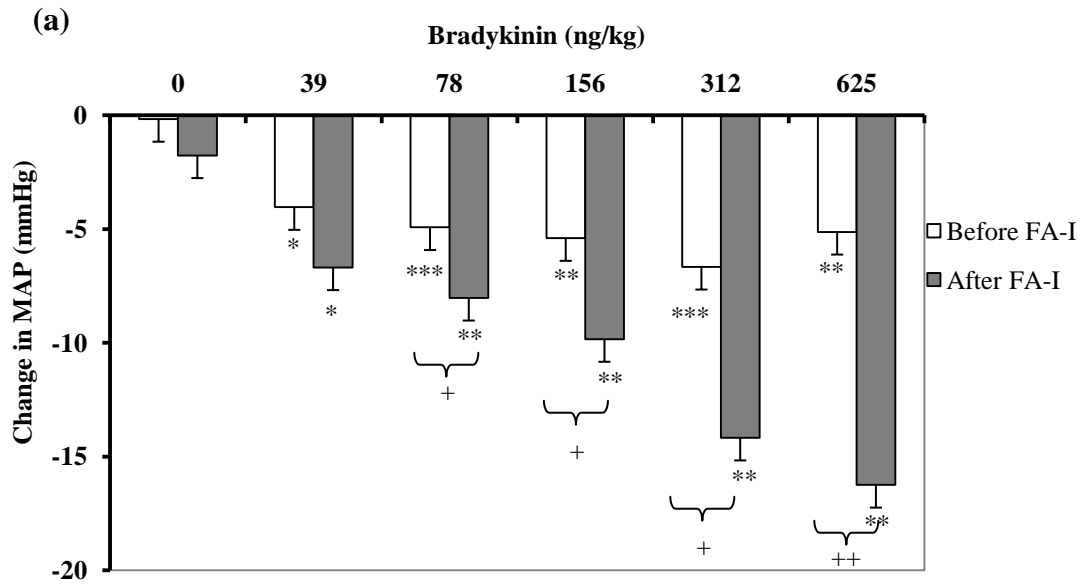


Figure 3.7 : Effect of intravenous bradykinin on the (a) mean arterial pressure (MAP) and (b) heart rate (HR) of anaesthetised rats before and after 10mg/kg FA-I administration. Values are mean \pm S.E.M. (n = 8). *p < 0.05; **p < 0.01; ***p < 0.001 compared with 0 ng/kg bradykinin and +p < 0.05; ++p < 0.01 compared with controls (before FA-I).

3.4 *In vitro* assays

3.4.1 *In vitro* effect of FA-I and its subfractions on angiotensin-converting enzyme activity

Figure 3.8 displays the effect of FA-I on ACE activity. Fraction FA-I was shown to inhibit ACE activity in a concentration dependent manner with an IC_{50} value of 0.52 mg/ml.

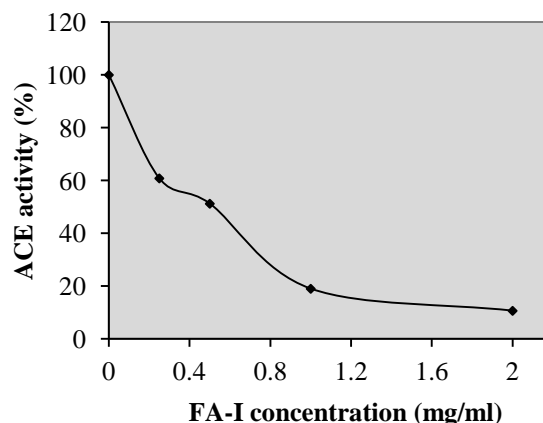


Figure 3.8 : Effect of a partially purified aqueous fraction (FA-I) on angiotensin-converting enzyme (ACE) activity. Data are means (duplicate assays).

Among the subfractions of FA-I, FA-Ia, FA-Ib and FA-Ic were found to have inhibitory effects on ACE activity by more than 50% at a concentration of 1.0 mg/ml each (Figure 3.9). The IC_{50} values for FA-Ia, FA-Ib and FA-Ic were 0.43, 0.95 and 0.58 mg/ml respectively (Table 3.2). Subfractions FA-Id and FA-Ie did not cause any obvious inhibition of ACE activity (Figure 3.9).

Table 3.2 : IC_{50} values of FA-I subfractions.

FA-I subfractions	IC_{50} (mg/ml)
FA-Ia	0.43
FA-Ib	0.95
FA-Ic	0.58
FA-Id	-
FA-Ie	-

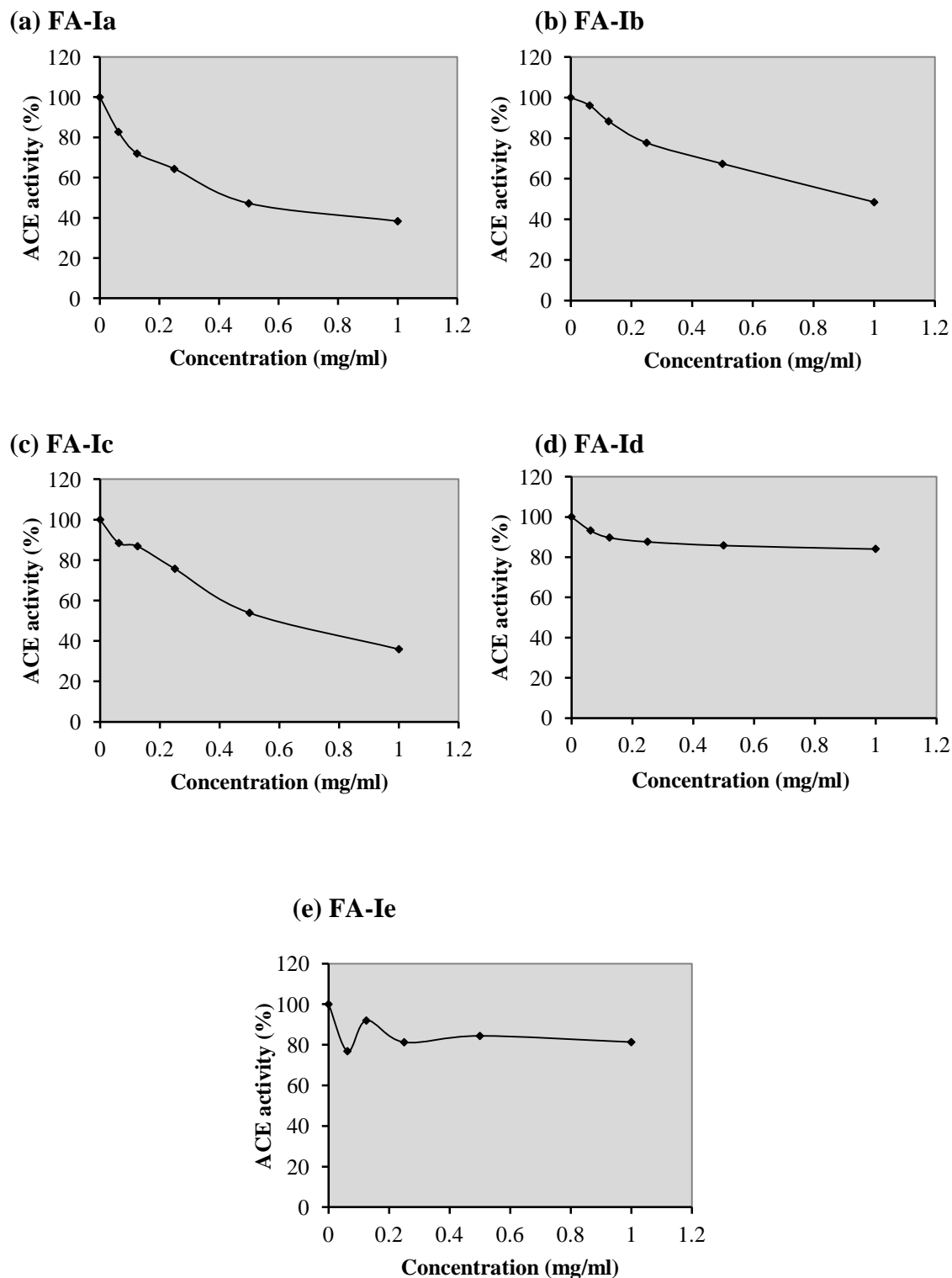


Figure 3.9 : Angiotensin-converting enzyme (ACE) inhibition by (a) FA-Ia, (b) FA-Ib, (c) FA-Ic, (d) FA-Id and (e) FA-Ie. Data are means (duplicate assays).

3.4.2 *In vitro* effect of FA-I and its subfractions on neutral endopeptidase activity

Figure 3.10 displays the effect of FA-I on NEP activity. Fraction FA-I was shown to inhibit NEP activity in a concentration dependent manner with an IC_{50} value of 225 $\mu\text{g/ml}$. Among the subfractions of FA-I, none of them shows obvious NEP inhibitory activity (Figure 3.11).

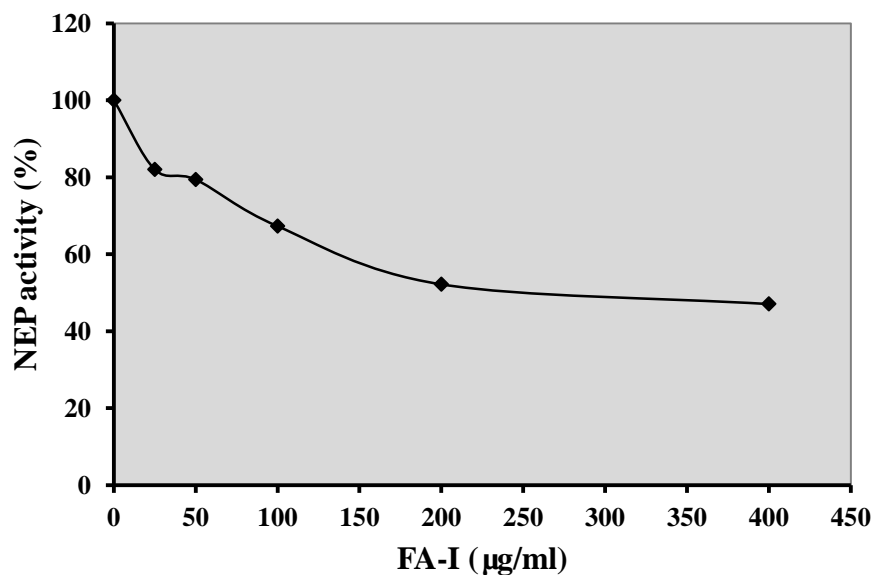
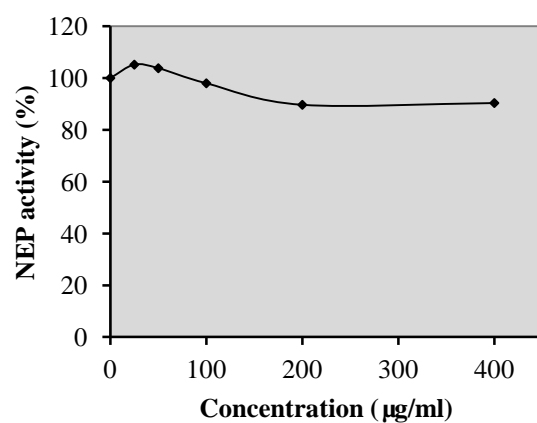
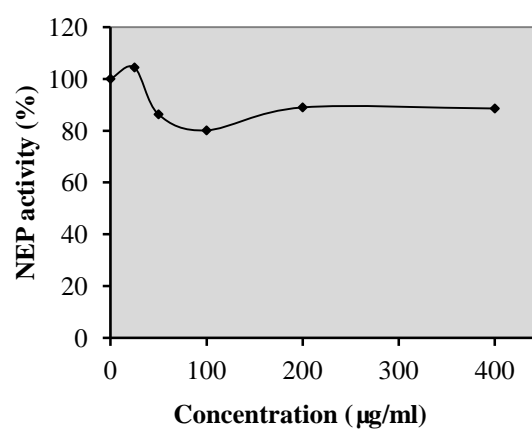


Figure 3.10 : Effect of a partially purified aqueous fraction (FA-I) on neutral endopeptidase (NEP) activity. Data are means (triplicate assays).

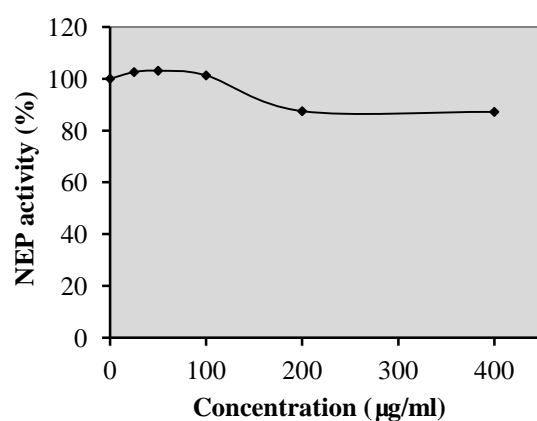
(a) FA-Ia



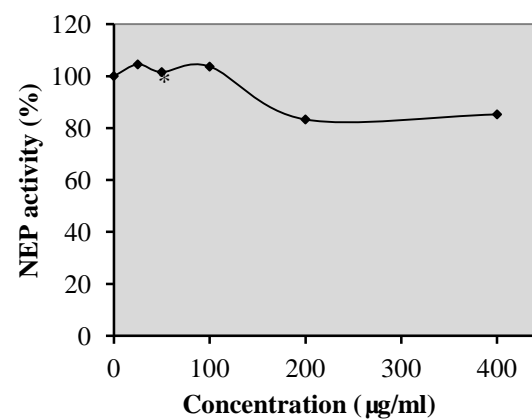
(b) FA-Ib



(c) FA-Ic



(d) FA-Id



(e) FA-Ie

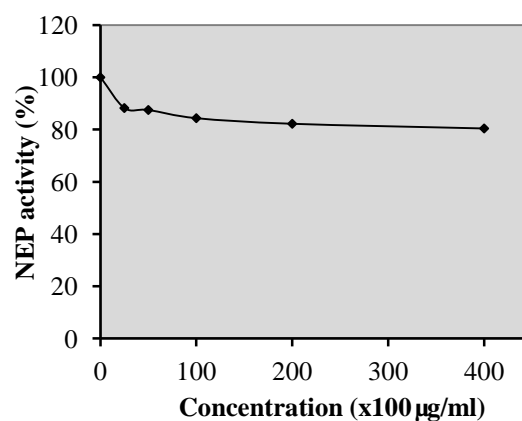


Figure 3.11 : Neutral endopeptidase (NEP) inhibition by (a) FA-Ia, (b) FA-Ib, (c) FA-Ic, (d) FA-Id and (e) FA-Ie. Data are means (triplicate assays).

3.5 Effect of orally administered FA-I on blood pressure of spontaneously hypertensive and Wistar-Kyoto rats

Figure 3.12 shows the time-course changes in the SBP of SHR and control WKY rats after a single oral administration of FA-I (50, 100 or 200 mg/kg) or vehicle (distilled water). An oral dose of FA-I was able to cause a significant ($p < 0.05$) dose-dependent decrease in the SBP especially at the higher doses when compared to controls (Figure 3.12). In the SHR rats, there is a tendency for the SBP to return to the initial level within 24 h after oral administration and the highest antihypertensive effect of FA-I occurred at 6 h after the oral administration (Figure 3.13).

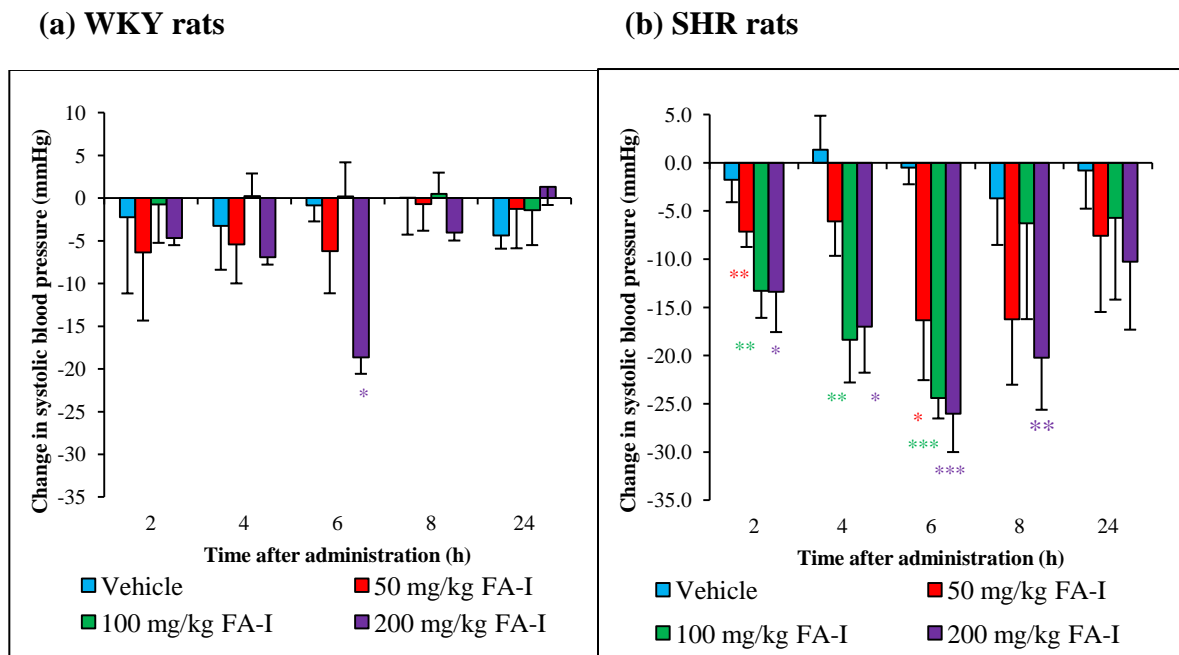


Figure 3.12 : Change in systolic blood pressure after a single orally administered of partially purified aqueous fraction (FA-I) in (a) Wistar-Kyoto (WKY) and (b) spontaneously hypertensive (SHR) rats. Values are mean \pm S.E.M. (WKY, $n = 6$; SHR, $n = 7$). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with vehicles (distilled water).

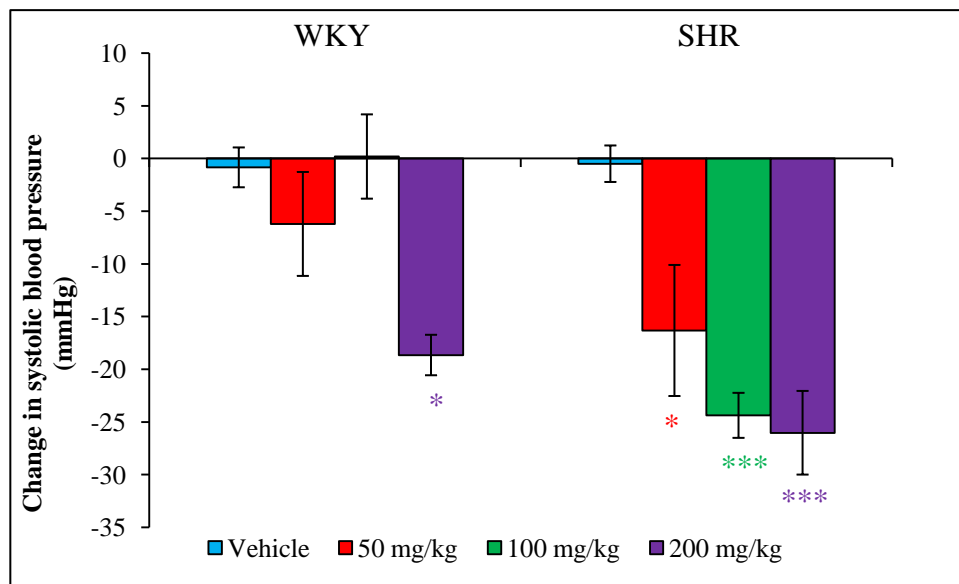


Figure 3.13 : Change in systolic blood pressure in Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats at 6 hours after a single orally administered of partially purified aqueous fraction (FA-I). Values are mean \pm S.E.M. (WKY, n = 6; SHR, n = 7). *p < 0.05, **p < 0.01 and ***p < 0.001 compared with vehicles (distilled water).

3.6 Characterisation of FA-I subfractions

Among the five subfractions of FA-I, only FA-Ia, FA-Ib and FA-Ic showed bioactive activity (Figure 3.9, Figure 3.11). However, when subjected to ^1H NMR spectrometer, no conclusive data could be observed for FA-Ia (Appendix E), FA-Ib (Appendix F) and FA-Ic (Appendix G). Nevertheless, when using other spectrometric analyses, a putative 16 carbon compound was obtained in FA-Ia.

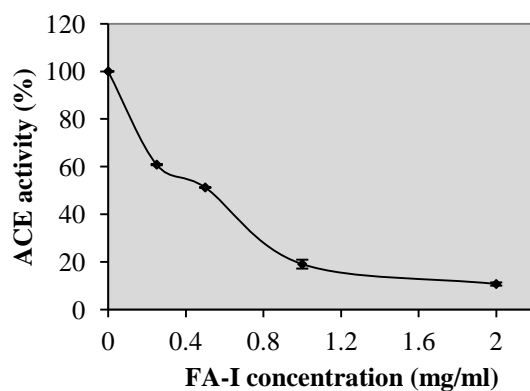
The structure of FA-Ia, elucidated by ^1H (Appendix E) and ^{13}C NMR (Appendix H) spectroscopies, shows the presence of aliphatic protons within chemical shifts δ 1~2 ppm, protons adjacent to an olefin and/or a carbonyl group within δ 3~4 ppm and olefinic protons within δ 5~6 ppm (by ^1H NMR) with a total of 16 carbons (by ^{13}C NMR). Four CH/CH₃, ten CH₂ and two quaternary carbons were revealed in the DEPT spectrum

(Appendix I). The mass spectrum data obtained from LC-MS showed a molecular ion at m/z 279 $[M + H]^+$ (Appendix J) suspected for $C_{16}H_{22}O_4$. Strong infrared absorptions for functional groups hydroxyl (OH), 3297 cm^{-1} ; aliphatic (C-H), 2923 and 2854 cm^{-1} ; carbonyl (C=O), 1740 cm^{-1} and ether (C-O), 1047 cm^{-1} stretching frequencies were found in the molecule (Appendix K). However, the definitive structure of FA-Ia remains unresolved.

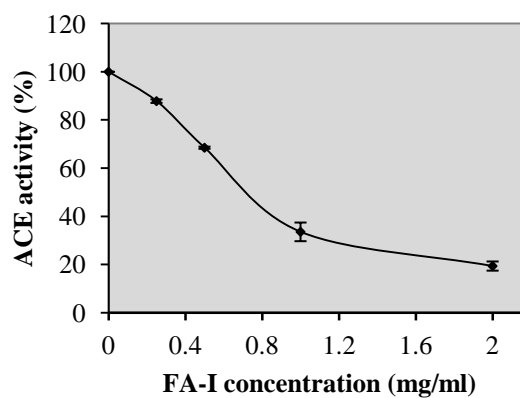
3.7 Effect of proteases on angiotensin-converting enzyme inhibitory activity of FA-I

Figure 3.14 shows the ACE inhibitory activity of FA-I without and after the treatment of proteases. Overall, the ACE inhibitory activity of FA-I was still preserved after the treatment of proteases. There was a slight decrease in ACE inhibitory activity of FA-I, as indicated by increase in their IC_{50} values, after pepsin, trypsin or chymotrypsin treatment (Table 3.3).

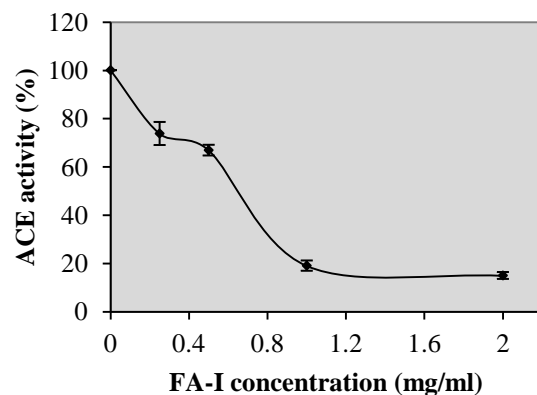
(a) Without treatment



(b) Pepsin treatment



(c) Chymotrypsin treatment



(d) Trypsin treatment

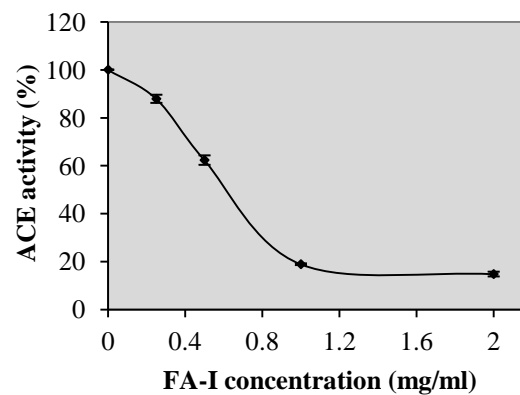


Figure 3.14 : The angiotensin-converting enzyme (ACE) inhibitory activity of FA-I (a) without and after (b) pepsin, (c) chymotrypsin and (d) trypsin. Data are means (duplicate assays).

Table 3.3 : The effect of digestive enzymes on the IC_{50} values of FA-I.

Digestion	IC_{50} of FA-I (mg/ml)
None	0.52
Pepsin	0.70
Trypsin	0.60
Chymotrypsin	0.64

CHAPTER 4 - DISCUSSION

4.1 *In vitro* vascular tension studies

In vitro vascular tension study was performed to extend previous studies that clearly demonstrated the BP-lowering effects of *G. procumbens* by inhibition of ACE activity (Hoe *et al*, 2007). Aortic rings were used to study the effect of FA-I on the contraction caused by Ang I and Ang II because ACE is abundantly present in the aortic rings (Andre *et al*, 1990; Egleme *et al*, 1990). As shown in Figure 3.3, preincubation with FA-I significantly inhibited the Ang I-induced vasocontraction in both endothelium-intact and -denuded aortic rings, with the degree of relaxation produced in endothelium-intact rings being significantly ($p < 0.05$) greater than that obtained in the denuded ones. These observations indicate that the actions of FA-I are more enhanced in the presence of the endothelium, probably because of the presence of NO (Sandoo *et al*, 2010) and PGI₂ (DalBo *et al*, 2008). The effects are likely to be mediated through the newly converted Ang II from Ang I by ACE that are abundantly found in aortic rings (Andre *et al*, 1990; Egleme *et al*, 1990) as Ang I *per se* appears to have no physiological function (Peach, 1977; Hayashi and Kimoto, 2010). This finding also further confirms the previous studies (Hoe *et al*, 2007) that FA-I inhibits ACE which then reduces the production of Ang II and causes a drop in the contractile response of Ang II.

Angiotensin II is a potent vasoconstrictor and acts mainly through the widely distributed AT₁R to increase BP (Lote, 2006). Increased RAS activity which leads to the increase in Ang II has been implicated as a major contributing factor in the development of hypertension (Navar *et al*, 2011). The present study shows that FA-I is able to inhibit the Ang II-induced contraction of aortic rings with endothelium (Figure 3.4a). Experiments were then carried out on endothelium-denuded aortic rings to investigate the involvement of endothelium on the effect of FA-I on Ang II-induced contraction of aortic rings. In

endothelium-denuded aortic rings, FA-I did not appear to have a significant effect on the contraction evoked by Ang II (Figure 3.4b). This may exclude the notion that FA-I could be an AT₁R blocker as AT₁R blockers are known to inhibit Ang II-induced contractions in endothelium-denuded aortic rings as well (d'Uscio *et al*, 1998; Watts *et al*, 1998). Hence, this strongly suggests that the inhibitory effect of FA-I on the vascular contraction is entirely endothelium dependent.

It is well known that the endothelium plays an important role in the regulation of vascular tone by releasing both relaxant and contractile factors in response to chemical and physical stimulation (Villar *et al*, 2006; DalBo *et al*, 2008; Sandoo *et al*, 2010). The vasorelaxant substances released by endothelial cells include NO mainly produced by eNOS (Sandoo *et al*, 2010) and PGI₂ produced by the COX pathway (DalBo *et al*, 2008). In the present study, pEC₅₀ value was used to study the influence of L-NAME and indomethacin because there is no significant difference in the E_{max} values among the aortic rings preincubated with L-NAME (Figure 3.5a) or indomethacin (Figure 3.5b). The inhibitory effect of FA-I on the Ang II-induced contraction of aortic rings was reduced in the presence of L-NAME (Figure 3.5a) or indomethacin (Figure 3.5b), with significant ($p < 0.01$) reduction in pEC₅₀ values (Table 3.1). A reduced pEC₅₀ value shows that the graph is shifted to the right, indicating that in the presence of FA-I, the contractile effect of Ang II is reduced and more Ang II is needed to cause the same level of contraction. These findings support the notion that FA-I is able to counteract the contractile response of Ang II by stimulating the release of vasorelaxing factors via both the eNOS and COX pathways. In the presence of L-NAME in which eNOS is inhibited, FA-I is able to decrease Ang II-induced contraction probably by triggering the release of PGI₂. Similarly, in the presence of indomethacin, FA-I may be able to stimulate the release of NO, despite COX being inhibited.

4.2 Effects of FA-I on bradykinin by *in vitro* and *in vivo* studies

Bradykinin is a vasodilator produced in the kallikrein-kinin system (Campbell, 2000) in which most of the effects of BK are being mediated by B₂ receptor under normal conditions (Nishiyama and Kim-Mitsuyama, 2010). Inhibition of ACE may partly potentiate the actions of BK, by either an indirect action on B₂ receptor (Deddish *et al*, 2002) or direct activation of B₁ receptor (Erdos, 1999) to confer additional benefits to the CVS. In order to study the effects of FA-I on hypotensive and vasodilatory effects of BK, both *in vitro* and *in vivo* methods with reference to the methods performed by Anozie *et al*, 2007 and Maia *et al*, 2004, with slight modifications.

In the *in vitro* study, BK evoked a dose-dependent relaxation of PE-induced contraction of aortic rings, similar to the study of that reported by Anozie *et al*, 2007. As in the *in vivo* study, BK produced a dose-dependent hypotensive effect, similar to the report by Maia *et al*, 2004. At the highest dose of BK (625 ng/kg), the drop in MAP is smaller, but not significant when compared to the previous dose (312 ng/kg). This may be due to the baroreceptor reflex (Guyton and Hall³, 2006) in the rats to prevent further drop in MAP. In this *in vivo* study, the MAP, which is the pressure that drives the blood into tissues during the cardiac cycle, was used as the parameter for BP comparison because it minimise the inaccuracies of SBP and DBP (Smulyan and Safar, 2011). The formula of MAP that used in this study was $MAP = DBP + 1/3(SBP - DBP)$ because it is the most common formula that is being used worldwide (Smulyan and Safar, 2011).

Studies shown here demonstrated that in the presence of FA-I, the vasorelaxant (*in vitro* study) and BP-lowering (*in vivo* study) effects of BK are greater. In the study on aortic rings in the presence of FA-I, the relaxation effect brought about by BK on PE-precontracted endothelium-intact aortic rings is increased (Figure 3.6). However, parallel experiments on endothelium-denuded rings were not performed as it is well-established

that vasodilation caused by BK is entirely endothelium dependent (Vanhoutte, 2001). In the *in vivo* studies, the BP-lowering effect of BK was also greater after administration of FA-I (Figure 3.7a). Although BK is known to cause bradycardia (Wang *et al*, 2003), the infusion of FA-I did not alter the bradycardic effect of BK (Figure 3.7b). This could be due to the baroreceptor reflex (Wang *et al*, 2003) being involved in preventing further reduction in the HR brought about by FA-I. Hence, FA-I is able to potentiate the hypotensive effect of BK probably by enhancing the vasodilator activities of BK through inhibition of ACE or NEP, but without alteration in HR.

Other than that, it is speculated that FA-I may potentiate the hypotensive effect by BK through the vasodilatory effect of Ang (1-7) which is increased after inhibition of ACE (Fernandes *et al*, 2001). The FA-I may also possess antioxidant effect that preserves the production of NO which is reduced due to oxidative stress produced by PE (Hao *et al*, 2006). Nevertheless, the exact mechanism responsible for the BK potentiation effects of FA-I remains to be elucidated.

4.3 Vasopeptidase inhibitory activities of FA-I and FA-I subfractions

One of the most popular VPIs, omapatrilat, had high incidence of life-threatening side-effect angioedema and was thus withdrawn by FDA (Song and White, 2001). Searching for potential VPIs from natural products may alleviate the side-effects, especially that of angioedema. Although VPI is deemed to be more superior to ACEI or NEPI alone, studies on potential dual ACE/NEP inhibitory activities from natural products have less popular attention. Examples of plants with dual ACE/NEP inhibitory activities are *Ligustrum vulgare* (Kiss *et al*, 2008) and *Epilobium angustifolium* (Kiss *et al*, 2006). Since FA-I was shown to have strong ACE inhibitory activity (Hoe *et al*, 2007), it would be interesting to investigate whether this plant has NEP inhibitory activity as well.

In studying the potential dual ACE/NEP inhibitory activities, spectrophotometric method (for measurement of ACE activity) and spectrofluorometric method (for measurement of NEP activity) were used. Both of these methods are volume sensitive and the enzymes are sensitive to the environment and need to be handled with care. The amount of FA-I and its subfractions used were referred to the previous study with little adjustment (Hoe *et al*, 2007). Micrograms of extracts were used in NEP activity study as reference to Kiss *et al*, 2008 (Kiss *et al*, 2008).

Result shows that FA-I has strong ACE inhibitory activities (Figure 3.8), which is similar to that reported by Hoe *et al*, 2007. Among the FA-I subfractions, FA-Ia, FA-Ib and FA-Ic inhibited ACE activities at 1.0 mg/ml (Figure 3.9). In this study, an ACEI drug, captopril (2.6 mg/ml) is able to inhibit 98.44% of ACE activity. The ACE inhibitory effect of FA-I and its bioactive subfractions seem to be comparable to captopril but no further conclusion can be made because only a single dose of captopril was used. Among the FA-I subfractions, FA-Ia may have the strongest ACE inhibitory activity because it has the lowest IC₅₀ value (Table 3.2). These findings show that some FA-I subfractions retain the

ACE inhibitory activity and the ACE inhibitory compounds that are present in FA-I may also present in these subfractions.

Besides the ACE inhibitory activity, our study successfully shows that FA-I possesses some NEP inhibitory activity, with IC_{50} value of 225 $\mu\text{g/ml}$ (Figure 3.10). The NEP inhibitory effect is far weaker when comparing to a single dose of phosphoramidon (100 $\mu\text{g/ml}$), a reference drug used in this study, which decreases 97.9% of NEP activity. This may be because FA-I is only a partially purified fraction but the phosphoramidon is a popular NEP inhibitor drug. However, this NEP inhibitory activity diminishes in FA-I subfractions (Figure 3.11). It is speculated that there may exist more than one NEP inhibitory compounds present in FA-I. In order to exhibit strong NEP inhibitory effect, these compounds need to act synergistically and the amount of NEP inhibitory compounds that are present in each FA-I subfraction may not be enough to react to give strong inhibitory effects (Colegate and Molyneux, 2008). Another postulation is the loss of bioactive compounds during separation step (Colegate and Molyneux, 2008). The presence of NEP inhibitory activity in FA-I but not in its subfractions could also be due to the existence of unknown compounds in FA-I that are not revealed at the TLC plate by ninhydrin reagent. It is also possible that FA-I subfractions may also act at the allosteric sites of the enzyme that give a conformational change in the enzyme to enable the binding of other subfractions.

The dual ACE/NEP inhibitory effect of FA-I may also be responsible for the potentiation of the hypotensive effect of BK. This is because the main enzymes responsible for the hydrolysis of BK are ACE and NEP and the inhibition of these enzymes would result in an increase in the level of BK to enhance vasodilation to cause a drop in BP (Sharma and Al-Sherif, 2011).

4.4 Effect of orally administered FA-I on blood pressure of rats

At present, there are many methods being used to measure the BP of rats. Among these methods, radiotelemetry is claimed to be the most efficient and accurate method in measuring the BP of rats (Mills *et al*, 2000; Van Vliet *et al*, 2000). Tail-cuff method was adopted in this study because this method has the main advantage of no surgical and anesthesia procedures which minimise the risk of infection.

In order to minimise false positive readings brought by changes in BP due to heating and restraining (Irvine *et al*, 1997), rats were subjected to same sized restrainers, same preheating time and heating temperature; and similar environment. Training of rats prior to the experimental procedures made the rats better at adapting to the restraining conditions and they remained calmer in the restrainers.

Some studies have shown that antihypertensive agents may lose their effects after being treated orally (Li *et al*, 2004). Thus, oral feeding of FA-I on SHR and WKY rats were performed in this study. The SHR rat was chosen because it is the most widely used hypertensive animal model and the incidence of developing hypertension is 100% (Yamori and Okamoto, 1973) whereas the normotensive WKY rats serve as controls for SHR rats (Kurtz and Morris, 1987).

A single oral administration of FA-I exerted antihypertensive effect on SBP in SHR rats, with the highest effect occurring at 6 h after the administration (Figure 3.12b). However, no significant suppression in SBP was found in normotensive WKY rats except for the highest concentration, 200mg/kg of FA-I (Figure 3.12a). This shows that FA-I has antihypertensive effect and a lower dose of FA-I may be safe to normotensive subjects. In order to cause antihypertensive effect after oral administration, the agents need to reach the blood circulation (Vermeirssen *et al*, 2004). Therefore, active substances in FA-I are believed to be able to reach the blood circulation in an active form after oral treatment.

4.5 Effect of proteases on angiotensin-converting enzyme inhibitory activity of FA-I and nuclear magnetic resonance studies on FA-Ia

The results show that FA-I is able to retain most of its ACE inhibitory activity after pepsin, trypsin and chymotrypsin treatment with little increase in IC₅₀ values (Table 3.3). This protease study indicates that the active compounds in FA-I may not be amino acid and they can withstand the effects by proteases.

Ninhydrin (2,2-dihydroxyindane-1,3-dione) is a chemical used to detect ammonia, primary and secondary amines. When reacting with these amines, a deep blue or purple colour stain is produced on the TLC. Hence, ninhydrin is commonly used to detect amines in peptides or amino acids (Carey *et al*, 2010). However, this ninhydrin staining test can only be used as a preliminary phytochemical screening method because not all the amine-containing compounds give positive results with ninhydrin and false positive results were also reported in non-amine-containing compounds (Carey *et al*, 2010). A more reliable structural characterisation method is NMR spectroscopy (Colegate and Molyneux, 2008). An amine-containing compound, i.e. amino acid could be detected explicitly by using ¹H-NMR and ¹³C-NMR. The ¹³C-NMR chemical shifts for an amino acid for example are in the range of 53.1 – 63.3 ppm (C-NH₂) and 174.1 – 177.6 ppm (COOH) (Appendix L) (Wishart and Nip, 1998). In the case of fraction FA-Ia, no amine or amino acid functional group was detected in the ¹H-NMR and ¹³C-NMR. Hence, FA-Ia is not an amino acid. This is contrary to the study by Hoe *et al*, 2007 who postulated that FA-I may be peptides.

4.6 General discussion and future studies

Hypertension is the most important risk factor for cardiovascular mortality and morbidity worldwide. Although many types of antihypertensive treatments are available either clinically or traditionally, the prevalence of hypertension remains high and keeps on increasing (Kim *et al*, 2010). Many studies have been carried out in search of novel antihypertensive treatments.

Natural products with great structural diversities have made them valuable sources for the discovery of novel antihypertensive drugs. The present study has shown some advance findings of the plant *G. procumbens*. It is found to be being able to inhibit significantly ($p < 0.05$) the contraction brought by Ang II, probably via activation of the release of NO and PGI₂ or it may act on an unknown receptor which is responsible for the contraction of Ang II. Future studies may be carried out to identify this receptor which may be present in the vasculars.

Bradykinin has been known to contribute to the cardioprotective effects of ACEIs (Murphey *et al*, 2003). Previous findings have shown that *G. procumbens* has strong ACE inhibitory activities (Hoe *et al*, 2007) and study was carried out to test whether this plant has effects on BK. Results presented here have shown that the BP-lowering effect of BK is enhanced which may be caused by the greater vasodilatory effect of BK in the presence of plant extract. One of the possible pathways other than the ACE and NEP inhibition, is activation of B₁ or B₂ receptors which can be explored in future.

Acute oral feeding studies showed that the antihypertensive effect of *G. procumbens* is still preserved after going through the gastrointestinal system. This is a great finding which gives clue that it may be effective when applied orally to the human. The possible adverse side-effects that may be arising have not studied and this remains to be explored.

Finally, in order to contribute to the antihypertensive treatments, the compounds are needed to be isolated, identified and produced industrially. It is necessary to identify the bioactive compounds that are responsible for the effects of *G. procumbens*, although one needs to take into account the heterogeneity of hypertension in order to evaluate the potential impact of treatment for the management of this disorder. The systems in the human body react differently to the hypertension and hypertensive patient react differently to the antihypertensive treatments. Side-effects of the drugs that are arising should not be ignored. Natural products represent the major source of chemical diversity for antihypertensive screening purposes and communication among the experts such as botanists, chemists, clinicians and natural products researchers should be well established so that the biodiversity that exists in nature is not lost for antihypertensive drug discovery.

CHAPTER 5 - CONCLUSIONS

This study conclusively demonstrates and extends previous investigations that FA-I, fractionated from the leaves of *G. procumbens*, has potent BP-lowering effect acting via dual ACE/NEP inhibitory activity as well as BK enhancement. Finding of FA-I being able to antagonise the vasoconstrictive effect of Ang II is reported. The effect of FA-I on Ang II-induced contraction of aortic rings appears to be mediated by an endothelium-dependent pathway that involves the activation of NO and COX mechanisms. In addition, FA-I is able to enhance the BK effect, most probably by stimulation of the release of NO and PGI₂. These findings are summarised in Figure 5.1.

As part of our search for potential VPI from plant, this report indicates that FA-I has dual ACE/NEP inhibitory activity. As far as can be ascertained, this is the first study that demonstrates the dual effect of *G. procumbens*. Although only FA-I and not its subfractions show NEP inhibitory activity, ACE inhibitory activities are shown by FA-Ia, Fa-Ib and FA-Ic (Figure 5.1).

This study also shows that FA-I has antihypertensive effect on SHR and little effect on WKY rats after it is being treated orally. It is also able to withstand proteases to maintain most of its ACE inhibitory activity. The NMR and HPLC-MS studies indicate that FA-Ia is a 16 carbon molecule suspected for C₁₆H₂₂O₄ that has OH, aliphatic C-H, C=O and C-O functional groups.

SUMMARY

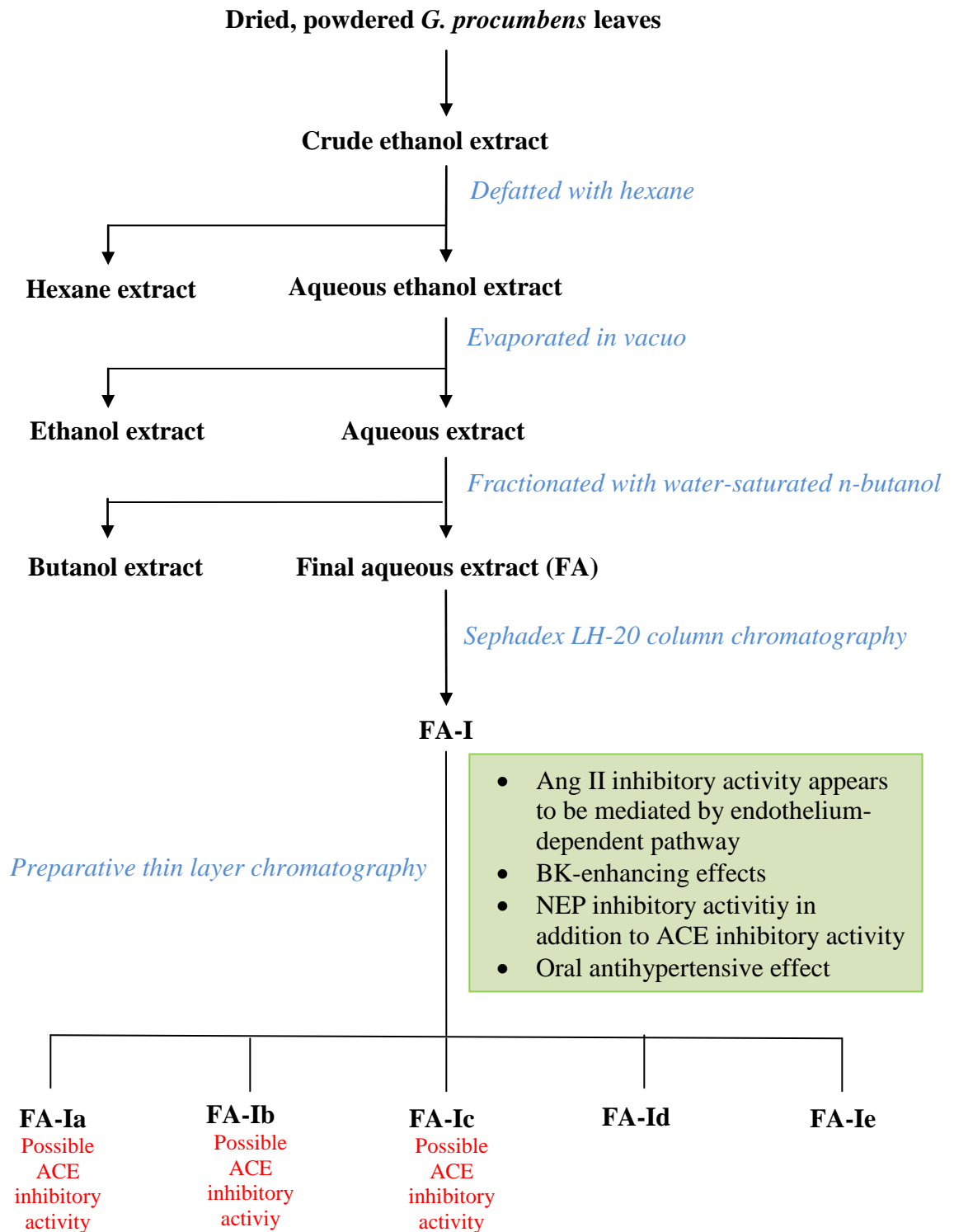


Figure 5.1 : Extraction and fractionation of *Gynura procumbens*, the effects and mechanisms of actions.

PUBLICATIONS

Papers

1. Poh T.F., Ng, H.K., Hoe, S.Z. and Lam, S.K. (2013) *Gynura procumbens* causes vasodilation by inhibiting angiotensin II and enhancing bradykinin actions. *Journal of Cardiovascular Pharmacology*. **61** (5): 378-384.
2. Poh T.F., Ng, H.K., Hoe, S.Z. and Lam, S.K. First report of dual inhibition of ACE and NEP by a partially purified fraction of *Gynura procumbens*. (*In preparation*).
3. Poh T.F., Chin, C.F., Hoe, S.Z. and Lam, S.K. Chemical analysis of an active compound of *Gynura procumbens* using LCMS and HPLC and NMR spectroscopy. (*In preparation*).

Abstract

1. Poh T.F., Ng, H.K., Hoe, S.Z. and Lam, S.K. Hypotensive effect of oral administration of putative ACE inhibitors from *Gynura procumbens*. *In proceeding: 24th Malaysian Society of Pharmacology and Physiology (MSPP) Scientific Meeting*. 2nd – 3rd June 2010. SACC Convention Centre, Shah Alam. (p. 125)

Gynura procumbens Causes Vasodilation by Inhibiting Angiotensin II and Enhancing Bradykinin Actions

Ting-Fung Poh, Hien-Kun Ng, See-Ziau Hoe, PhD, and Sau-Kuen Lam, PhD

Abstract: Previous studies showed that *Gynura procumbens* reduced blood pressure by blocking calcium channels and inhibiting the angiotensin-converting enzyme activity. The present experiments were to further explore the effects and mechanisms of a purer aqueous fraction (FA-I) of *G. procumbens* on angiotensin I (Ang I)-induced and angiotensin II (Ang II)-induced contraction of aortic rings and also on the bradykinin (BK) effect on cardiovascular system. Rat aortic rings suspended in organ chambers were used to investigate the vascular reactivity of FA-I. Effect of FA-I on BK was studied by in vitro and in vivo methods. Results show that FA-I significantly ($P < 0.05$) decreased the contraction evoked by Ang I and Ang II. In the presence of indomethacin (10 μ M) or N^ω-nitro-L-arginine methyl ester (0.1 μ M), the inhibitory effect of FA-I on Ang II-induced contraction of aortic rings was reduced. Besides, FA-I potentiated the vasorelaxant effect and enhanced the blood pressure-lowering effect of BK. In conclusion, FA-I reduced the contraction evoked by Ang II probably via the endothelium-dependent pathways, which involve activation of the release of nitric oxide and prostaglandins. The inhibition of angiotensin-converting enzyme activity by FA-I may contribute to the potentiation of the effects of BK on cardiovascular system.

Key Words: *Gynura procumbens*, angiotensin-converting enzyme, angiotensin II, bradykinin

(*J Cardiovasc Pharmacol*™ 2013;61:378–384)

INTRODUCTION

Normal blood pressure (BP) is partly maintained by the renin-angiotensin system (RAS) that regulates total sodium content and hence extracellular fluid volume.¹ One of the key enzymes in the RAS is angiotensin-converting enzyme (ACE). The pathway in which ACE splits 2 amino acids from the inactive decapeptide, angiotensin I (Ang I), to the octapeptide, angiotensin II (Ang II), that causes vasoconstriction, aldosterone release, sodium and water retention,¹ and increasing oxidative stress² is well-known. However, less studied is that ACE also metabolizes bradykinin (BK), a vasodilator

produced in the kinin-kallikrein system.³ Consequently, inhibition of ACE decreases both the formation of Ang II and hydrolysis of BK that subsequently brings about increased diuresis and a fall in BP.⁴ Therefore, inhibition of ACE is considered to be an effective therapeutic approach in treating hypertension. Most of the clinically used ACE inhibitors such as captopril, enalapril, and ramipril are effective not only in reducing BP but also in conferring beneficial effects on the cardiovascular system that seem to act mainly through the RAS.^{5–7} Moreover, most of the said ACE inhibitors are with side effects that range from mild to serious.⁸ Thus, exploration for new alternatives particularly in the plant kingdom as replacements for these drugs may provide invaluable findings.

Gynura procumbens Merr. (Compositae), a fast growing herbaceous plant, is used frequently in folk medicine for treating kidney diseases, fever, and rashes⁹ and for hypertension. Previous studies from our laboratory have shown that extracts from this plant are able to reduce BP by blocking calcium channels¹⁰ and inhibiting ACE activity,¹¹ besides being capable of lowering plasma lipids¹² and inhibiting renal mesangial cell proliferation.¹³ However, these studies were performed using relatively less purified extracts. Moreover, in view of the fact that Ang II increases BP mainly by vasoconstriction and ACE is involved in the degradation of BK, this study is thus initiated to explore further the effects and mechanisms of action of a purer aqueous fraction (FA-I) of *G. procumbens* on vasoreactivity and BP.

MATERIALS AND METHODS

All studies on experimental animals have been conducted under protocols reviewed and approved by the Animal Ethics Committee, University of Malaya Medical Centre.

Plant Materials

Whole plants of *G. procumbens*, excluding the roots, were collected from Peninsular Malaysia with a voucher specimen (KLU 047690) being deposited in the Herbarium at "Rimba Ilmu," University of Malaya.

Extraction and Fractionation of Plant Material

Leaves from fresh plants were cleaned and dried in an oven at 40°C and then ground to powder. A crude ethanolic extract was obtained by macerating the powder with 96% ethanol at room temperature for 72 hours. After that, the extract was concentrated to dryness in vacuo, and this resulted in a gummy dark residue that was then reconstituted in 80% aqueous ethanol. The resulting solution was partitioned with hexane in a separating funnel. The lower aqueous ethanolic layer was collected and subjected to evaporation in vacuo to remove the ethanol but

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From the Department of Physiology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia.

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The authors have no conflict of interest to declare.

Reprints: Sau-Kuen Lam, PhD, Department of Physiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia (e-mail: lam@ummc.edu.my).

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leaving behind an aqueous solution that contained an ethanol-soluble precipitate that was subsequently filtered out. The precipitate-free aqueous solution was then further partitioned against water-saturated n-butanol. The aqueous phase from this partition was collected and lyophilized to obtain the final aqueous fraction, which was further purified by passing it through a column containing Sephadex LH-20 gel using water as the eluent resulting in a much purer fraction, the FA-I fraction.

Animals

Adult male albino Sprague-Dawley rats, weighing between 250 and 320 g, were obtained from the Experimental Animal Care Unit, University of Malaya, and kept under standard conditions. Normal commercial rat chow (Gold Coin Animal Feed, Klang, Malaysia) and tap water were provided ad libitum.

In Vitro Experiments: Studies on Isolated Rat Thoracic Aortic Rings

Preparation of Rat Aortic Rings

The rats were sacrificed by decapitation, and the descending thoracic aorta was rapidly isolated and placed in a petri dish containing ice-cold oxygenated Krebs-Henseleit solution of the following composition (in mM): NaCl, 118.0; KCl, 4.7; CaCl₂, 1.25; MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 24.9; and glucose, 11.1. The aorta was cleaned of connective and adipose tissues and then cut into rings of 1–3 mm in length. Each aortic ring was mounted in an organ bath containing 10 mL Krebs-Henseleit solution with 2 stainless steel hooks as follows: the lower hook was fixed to the bottom of the bath, whereas the upper hook was connected to a force-displacement transducer to measure changes in the isometric contractile force that were recorded by a computerized physiograph system (PowerLab computer system; AD Instruments Pty. Ltd., Bella Vista, Australia). The bath solutions were maintained at a temperature of 37°C and bubbled continuously with 95% oxygen and 5% carbon dioxide gas mixture. The aortic rings were subjected to a resting tension of 1 g and allowed to equilibrate for 60 minutes although being rinsed every 15 minutes. Before each experiment, the aortic rings were stimulated at least 3 times with 6×10^{-2} M KCl until reproducible contractile responses were obtained.

Endothelium-denuded rings were prepared by inserting a pair of fine forceps into the lumen and gently rotating the rings around the forceps. These endothelium-denuded rings were similarly equilibrated in the organ baths for 60 minutes. The success of endothelial denudation was tested by precontracting the rings with phenylephrine (PE; 1×10^{-6} M) and observing the response to acetylcholine (1×10^{-5} M). A lack of any relaxation of the PE-precontracted rings indicated that the endothelium was satisfactorily removed, whereas relaxation of at least 70% of the PE-induced contraction showed that the endothelial layer was still present.

Effects of FA-I Pretreatment on Ang I-Induced Contraction

Endothelium-intact and endothelium-denuded aortic rings were preincubated for 20 minutes in the absence or presence of FA-I (1.0×10^{-4} and 1.0×10^{-3} g/mL). After the incubation period, contraction of the rings was evoked by adding cumulative

concentrations of Ang I ($1 \times 10^{-8.5}$ M to 1×10^{-6} M), and their responses in the absence or presence of FA-I were recorded.

Effects of FA-I Pretreatment on Ang II-Induced Contraction

Aortic rings were preincubated for 20 minutes in the absence or presence of FA-I (1.0×10^{-4} and 1.0×10^{-3} g/mL). After the incubation period, contraction of the rings was evoked by adding cumulative concentrations of Ang II ($1 \times 10^{-8.5}$ M to 1×10^{-6} M), and their responses in the absence or presence of FA-I were recorded.

Effects of FA-I Pretreatment on Ang II-Induced Contraction of Aortic Rings in the Presence of N^ω-nitro-L-arginine Methyl Ester or Indomethacin

To study the involvement of endothelium in response to Ang II brought by FA-I, endothelium-intact rings were preincubated with or without FA-I and indomethacin (10 μM), a nonselective cyclooxygenase (COX) inhibitor, or N^ω-nitro-L-arginine methyl ester (L-NAME; 0.1 μM), an endothelial nitric oxide synthase (eNOS) inhibitor, and followed by the cumulative additions of Ang II.

Effects of FA-I Pretreatment on BK-Induced Relaxation of PE-Precontracted Aortic Rings

After the equilibration period, the aortic rings were precontracted again with PE (1×10^{-6} M). The contractions of aortic rings to PE were allowed to reach a plateau before the cumulative additions of BK (1×10^{-11} M to 1×10^{-6} M). The relaxation responses of PE-precontracted aortic rings to the cumulative additions of BK were determined in the presence or absence of pretreatment with FA-I (1.0×10^{-4} and 1.0×10^{-3} g/mL) 20 minutes before the subsequent sequential additions of PE and BK.

In Vivo Experiments: Effects of FA-I on the Action of BK on Mean Arterial Pressure and Heart Rate of Rats

Rats were anaesthetized with sodium pentobarbitone (60%, wt/vol), 50 mg/kg, by intraperitoneal injections. Each rat was placed on a surgical board, and the right jugular vein, left carotid artery, and trachea were surgically exposed. Heparinized polyethylene tubing was inserted into the carotid artery for continuous monitoring of the BP via a pressure transducer connected to the PowerLab computer system. The jugular vein was cannulated with a heparinized polyethylene tubing for intravenous injections of the test solutions, whereas the trachea was cannulated to facilitate spontaneous respiration. Small animal electrographic leads were attached to the limbs of the animal to monitor the heart rate (HR), with the running electrocardiogram being recorded from lead II. The animal was kept warm ($35 \pm 2^\circ\text{C}$) with a heating lamp throughout the experiment. The setup was allowed to equilibrate for at least 30 minutes before the commencement of the experiments.

After the equilibration period, intravenous injections of BK at different doses (0–625 ng/kg) were done before and after FA-I (10 mg/kg) administration. A minimum interval of 3 minutes was allowed between each injection. Changes in

the BP due to the administration of BK and FA-I were obtained by calculating the difference between the BP before and the lowest BP after the injections. The mean arterial pressure (MAP) was calculated by using the following formula: $MAP = DBP + 1/3(SBP - DBP)$, where DBP is diastolic BP and SBP being systolic BP.

Chemicals and Drugs

All drugs and chemicals were obtained from Sigma Chemical Co. (St Louis, MO) except heparin (Leo Pharmaceutical Products; Ballerup, Denmark) and BK and Ang I (human) (Merck KGaA; Darmstadt, Germany). All chemicals and solvents were of analytical grade.

Data Analysis

When the cumulative concentration–response curves with Ang I and Ang II were compared, the contractions in the presence of FA-I were calculated as a percentage of the highest contractile response obtained in the absence of FA-I. This contractile response evoked by the highest concentration of Ang I or Ang II was designated as E_{max} . The pEC_{50} value ($-\log EC_{50}$) determined from the EC_{50} value, which is the concentration required to produce a half-effect in the concentration–response curves, was calculated from individual log concentration–response curves of the Ang I or Ang II by sigmoidal nonlinear regression analysis within the 95% confidence intervals using GraphPad Prism v.4.00 (GraphPad Software Inc., La Jolla, CA). The vasorelaxant response to BK was measured from the plateau of the PE-induced contraction and expressed as a percentage relaxation of the PE-induced contraction.

Statistical Analysis

All values are expressed as mean \pm SEM for n number of rats or separate experiments. Statistical differences were evaluated by Student's t test. A probability level of less than 0.05 ($P < 0.05$) was considered to be significantly different.

RESULTS

In Vitro Experiments: Studies on Isolated Rat Thoracic Aortic Rings

Effects of FA-I Pretreatment on Ang I-Induced Contraction

Pretreatment with FA-I at 1.0×10^{-4} and 1.0×10^{-3} g/mL inhibited the Ang I-induced contraction of both endothelium-

intact (Fig. 1A) and endothelium-denuded (Fig. 1B) aortic rings in a concentration-dependent manner with significant ($P < 0.01$) reduction in the E_{max} values. Furthermore, rings that were kept intact relaxed significantly ($P < 0.05$) more than those that were denuded of endothelium.

Effects of FA-I Pretreatment on Ang II-Induced Contraction

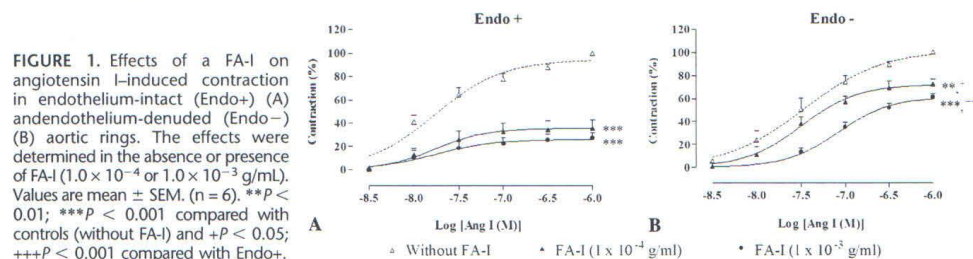
Experiments were then carried out with endothelium-intact and endothelium-denuded aortic rings to study the involvement of endothelium in the response of FA-I to the contraction evoked by Ang II. Results show that pretreatment of endothelium-intact aortic rings with FA-I attenuated the contraction caused by Ang II (Fig. 2A), with the E_{max} values being significantly ($P < 0.001$) decreased when compared with controls. However, exposure of endothelium-denuded aortic rings to FA-I for 20 minutes before the addition of increasing concentrations of Ang II did not blunt the contractions induced by Ang II (Fig. 2B).

Effects of FA-I Pretreatment on Ang II-Induced Contraction of Aortic Rings in the Presence of L-NAME or Indomethacin

To further study the role of endothelial factors in the action of FA-I, Ang II-induced contraction in endothelium-intact rings were performed in the presence of both FA-I and L-NAME or indomethacin. Results show that pretreatment of FA-I at 1.0×10^{-4} and 1.0×10^{-3} g/mL in the presence of L-NAME (Fig. 3A) or indomethacin (Fig. 3B) did not cause any significant changes in the E_{max} values for Ang II as compared with controls.

In aortic rings pretreated with L-NAME, although there were no significant changes in E_{max} values, the concentration–response curves of Ang II are shifted to the right (Fig. 3A) with a significant ($P < 0.05$) decrease in pEC_{50} values (Table 1). Similarly, preincubation of FA-I and indomethacin also shifted the concentration–response curve to the right (Fig. 3B), with a significant ($P < 0.01$) reduction of pEC_{50} values for Ang II in the presence of FA-I at 1.0×10^{-3} g/mL (Table 1). These results show that the eNOS and COX pathways may be involved in the response of FA-I on Ang II-induced contraction of aortic rings.

Figure 4 shows the comparison of the E_{max} values obtained for Ang II-induced contraction of aortic rings preincubated with FA-I in the presence or absence of L-NAME or indomethacin. Preincubation of aortic rings with FA-I



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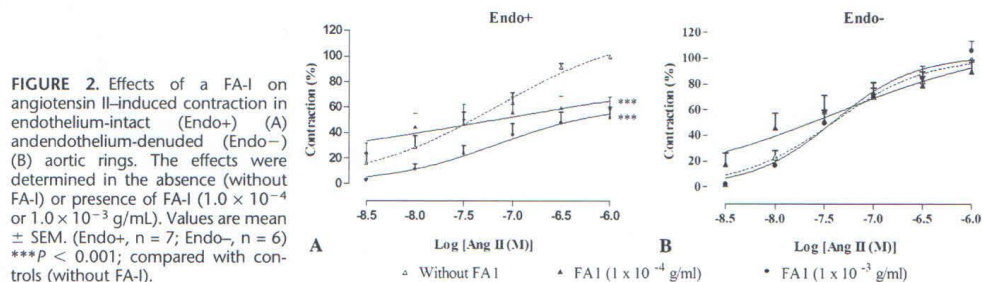


FIGURE 2. Effects of a FA-I on angiotensin II-induced contraction in endothelium-intact (Endo+) (A) and endothelium-denuded (Endo-) (B) aortic rings. The effects were determined in the absence (without FA-I) or presence of FA-I (1.0×10^{-4} or 1.0×10^{-3} g/mL). Values are mean \pm SEM. (Endo+, $n = 7$; Endo-, $n = 6$) *** $P < 0.001$; compared with controls (without FA-I).

and L-NAME produced a significantly ($P < 0.05$) higher E_{\max} value than the controls (FA-I and Ang II). For preincubation of aortic rings with FA-I and indomethacin, only the higher concentration of FA-I, which is 1.0×10^{-3} g/mL possesses significant ($P < 0.05$) higher E_{\max} value than the controls. In addition, at the lower dose, the abolishment of the vasodilatory effect due to FA-I by L-NAME is more significant ($P < 0.05$) than that observed for indomethacin.

Effects of FA-I Pretreatment on BK-Induced Relaxation of PE-Precontracted Aortic Rings

Bradykin (BK) (1×10^{-11} to 1×10^{-6} M) dose-dependently relaxed PE-induced contraction of aortic rings. This observation was significantly ($P < 0.05$) enhanced by pretreatment of aortic rings with FA-I (Fig. 5).

In Vivo Experiments: Effects of FA-I on the Action of BK on MAP and HR of Rats

Intravenous administrations of BK at different doses induced immediate and significant ($P < 0.05$) decrease in the MAP (Fig. 6A) and HR (Fig. 6B) of rats. After pretreatment with 10 mg/kg FA-I, the BP-lowering effect produced by BK was markedly ($P < 0.05$) greater. The effect of lowest concentration of BK, 39 ng/kg (-6.7 ± 1.5 mmHg) after FA-I administration was more pronounced than the effect produced by highest concentration of BK, 625 ng/kg (-5.1 ± 1.3 mmHg) before FA-I administration. The difference in the hypotensive effect of BK before and after FA-I pretreatment became significant ($P < 0.05$) at doses of 78,

156, 312, and 625 ng/kg. BK also decreased the HR, but there was no significant difference in the bradycardic effect between before and after FA-I administration. Predose baseline levels were recovered within 10 minutes after BK administration.

DISCUSSION

This study confirms and extends previous work that clearly demonstrated the BP-lowering effects of the plant, *G. procumbens*, by inhibition of ACE activity.¹¹ Furthermore, the results of this investigation also provide convincing evidence for a novel vascular response of FA-I, that is, it is able to antagonize the vasoconstrictive effect of Ang II.

As shown in Figure 1, preincubation with FA-I significantly inhibited the Ang I-induced vasocontraction in both endothelium-intact and endothelium-denuded aortic rings, with the degree of relaxation produced in endothelium-intact rings being significantly ($P < 0.05$) greater than that obtained in the denuded ones. These observations indicate that the actions of FA-I are more enhanced in the presence of the endothelium, probably because of the presence of nitric oxide (NO)¹⁴ and prostacyclin (PGI₂).¹⁵ The effects are likely to be mediated through the newly converted Ang II from Ang I by ACE that are abundantly found in aortic rings^{16,17} as Ang I per se seems to have no physiological function.^{18,19}

Angiotensin II is a potent vasoconstrictor and acts mainly through the widely distributed Ang II type 1 receptor (AT₁R) to increase BP.¹ Increased RAS activity, which leads

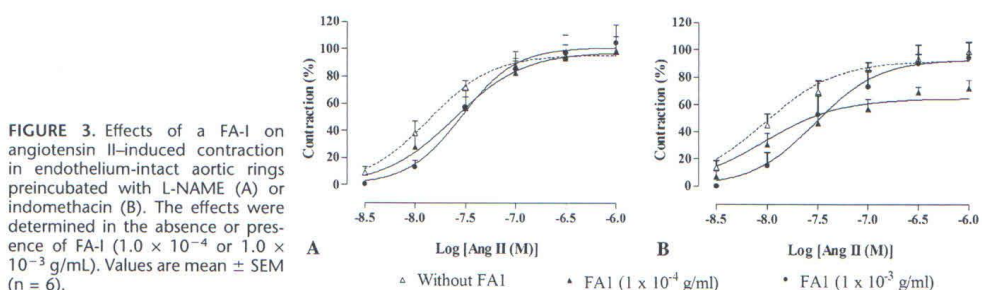


FIGURE 3. Effects of a FA-I on angiotensin II-induced contraction in endothelium-intact aortic rings preincubated with L-NAME (A) or indomethacin (B). The effects were determined in the absence or presence of FA-I (1.0×10^{-4} or 1.0×10^{-3} g/mL). Values are mean \pm SEM ($n = 6$).

TABLE 1. Effects of a FA-I on the pEC₅₀ Values for Ang II-Induced Contraction of Aortic Rings Pretreatment With L-NAME or Indomethacin

FA-I ($\times 10^{-3}$ g/mL)	pEC ₅₀	
	L-NAME	Indomethacin
0	7.84 \pm 0.06	7.89 \pm 0.08
0.1	7.61 \pm 0.12*	7.75 \pm 0.16
1.0	7.54 \pm 0.11†	7.53 \pm 0.14†

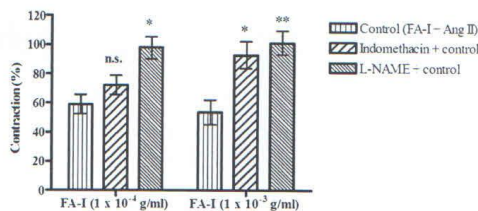
Values are mean \pm SEM (n = 6).

*P < 0.05.

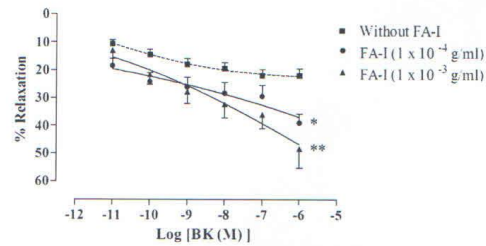
†P < 0.01 compared with controls (without FA-I).

to the increase in Ang II, has been implicated as a major contributing factor in the development of hypertension.²⁰ The present study shows a novel finding that FA-I is able to inhibit the Ang II-induced contraction of aortic rings with endothelium (Fig. 2A). Experiments were then carried out on endothelium-denuded aortic rings to investigate the involvement of endothelium on the effect of FA-I on Ang II-induced contraction of aortic rings. In endothelium-denuded aortic rings, FA-I did not seem to have a significant effect on the contraction evoked by Ang II (Fig. 2B). This may therefore exclude the notion that FA-I could be an AT₁R blocker as AT₁R blockers are known to inhibit Ang II-induced contractions in endothelium-denuded aortic rings as well.^{21,22} Hence, this strongly suggests that the inhibitory effect of FA-I on the vascular contraction is entirely endothelium dependent.

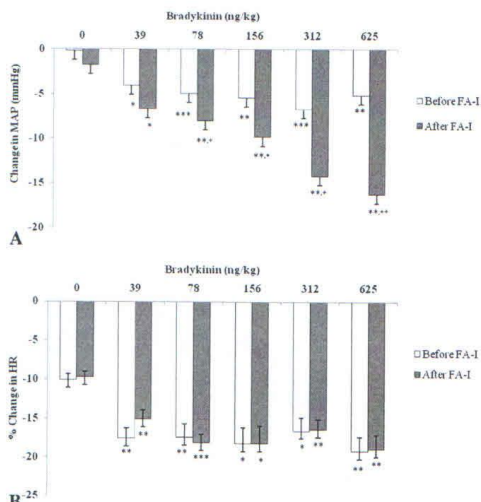
It is well known that the endothelium plays an important role in the regulation of vascular tone by releasing both relaxant and contractile factors in response to chemical and physical stimulation.^{14,15,23} The vasorelaxant substances released by endothelial cells include NO mainly produced by eNOS,¹⁴ PGI₂ produced by the COX pathway,¹⁵ and endothelium-derived hyperpolarizing factor.²³ In the present study, the inhibitory effect of FA-I on the Ang II-induced contraction of aortic rings was diminished in the presence of L-NAME (Fig. 3A) or indomethacin (Fig. 3B), with significant (P < 0.01) reduction in pEC₅₀ values (Table 1) but no significant

**FIGURE 4.** Comparison of the highest E_{max} values for respective FA-I on angiotensin II-induced contraction in aortic rings preincubated with or without L-NAME or indomethacin. The effect was determined in the absence or presence of L-NAME or indomethacin. Values are mean \pm SEM (control, n = 7; others, n = 6). NS, not significant; *P < 0.05; **P < 0.01 compared with controls.

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**FIGURE 5.** Effects of FA-I on BK-induced relaxation of aortic rings precontracted with PE. The effects were determined in the absence (without FA-I) or presence of FA-I (1.0×10^{-4} or 1.0×10^{-3} g/mL). Values are mean \pm SEM. (n = 8). *P < 0.05 **P < 0.01 compared with controls (without FA-I).

difference in E_{max} values. These novel findings support the notion that FA-I is able to counteract the contractile response of Ang II by stimulating the release of vasorelaxing factors via both the eNOS and COX pathways. Furthermore, in the presence of L-NAME in which eNOS is inhibited, FA-I is able to prevent the Ang II-induced contraction by triggering the release of PGI₂ (Fig. 3A). Similarly, in the presence of indomethacin, FA-I is able to stimulate the release of NO, despite COX being inhibited (Fig. 3B). In addition, preincubation of aortic rings with L-NAME caused a greater loss of the vasorelaxant response of FA-I than that of aortic rings preincubated

**FIGURE 6.** Effects of intravenous BK on the MAP (A) and HR (B) of anesthetized rats before and after 10 mg/kg FA-I administration. Values are mean \pm SEM (n = 8). *P < 0.05; **P < 0.01; ***P < 0.001 compared with 0 ng/kg BK and +P < 0.05; ++P < 0.01 compared with controls (before FA-I).

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with indomethacin (Fig. 4). These findings clearly indicate and support the data from Figure 2 that FA-I attenuates the Ang II-induced vasoconstriction mainly through the endothelium-dependent pathway with NO rather than PGI₂ playing a major role.

Apart from the effect of FA-I on Ang I-induced and Ang II-induced contractions, effect of FA-I on the relaxation response to BK was also studied (Fig. 5). BK is a vasodilator produced in the kinin-kallikrein system⁵ wherein 2 types of receptors have been defined as follows: B₁ and B₂ receptors with most of the effects of BK being mediated by B₂ receptor under normal conditions.⁵ Inhibition of ACE may partly potentiate the actions of BK, by either an indirect action on B₂ receptor or direct activation of B₁ receptor,^{24–27} and thus confer additional benefits to the cardiovascular system. Studies shown here demonstrated that FA-I does indeed enhance the vasorelaxant (in vitro study) and BP-lowering (in vivo study) effects of BK. The concentration of FA-I (10 mg/kg) used has no effect on the PE-induced contraction of aortic rings before cumulative addition of BK, as there was no significant difference in the magnitude of the PE-induced contraction in the presence or absence of FA-I (data not shown). In the study on aortic rings, FA-I enhanced the relaxation effect brought about by BK on PE-precontracted endothelium-intact aortic rings (Fig. 5). However, parallel experiments on endothelium-denuded rings were not performed as it is well established that vasodilation caused by BK is entirely endothelium dependent.^{28,29} In the in vivo studies, the BP-lowering effect of BK was enhanced by FA-I (Fig. 6A). Although BK is known to cause bradycardia,³⁰ the infusion of FA-I did not alter the bradycardic effect of BK (Fig. 6B). This could be due to the baroreceptor reflex^{31,32} being involved in preventing further reduction in the HR brought about by FA-I. Hence, FA-I is able to potentiate the hypotensive effect of BK by enhancing the vasodilator activities through inhibition of ACE or by indirect action on the BK B₂ receptor, but without alteration in HR. Nevertheless, the exact mechanism responsible for the BK potentiation effect of FA-I remains to be elucidated.

CONCLUSIONS

This study conclusively demonstrates and extends previous investigations that FA-I, fractionated from the leaves of *G. procumbens*, has potent ACE-inhibiting activity. In addition, a novel finding of FA-I being able to antagonize the vasoconstrictive effect of Ang II is reported. The effect of FA-I on Ang II-induced contraction of aortic rings seems to be mediated by an endothelium-dependent pathway that involves the activation of NO and COX mechanisms. Furthermore, FA-I is able to enhance the BK effect, most probably by stimulation of the release of NO and PGI₂. Therefore, FA-I could be developed into a more superior form of treatment for hypertension with dual effects than the conventional ones available now.

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HYPOTENSIVE EFFECT OF ORAL ADMINISTRATION OF PUTATIVE ACE INHIBITORS FROM *GYNURA PROCUMBENS*

Poh Ting Fung, Ng Hien Kun, Hoe See Ziau and Lam Sau Kuen
Department of Physiology, Faculty of Medicine, University of Malaya

Introduction: Previous studies have shown that a purer aqueous fraction (FA-I) of the leaves of *Gynura procumbens* contains angiotensin-converting enzyme (ACE) inhibitors that may, in part, contribute to the hypotensive effect observed. Besides, the method of blood pressure (BP) measurement used in the previous studies was that of direct technique that is invasive in nature. This investigation is therefore initiated to study the acute effect of oral administrations of FA-I in rats, and to use an indirect and non-invasive method of BP monitoring that enables repeated live- measurements.

Method: Spontaneously hypertensive (SHR; $n=6$) and normotensive Wistar-Kyoto (WKY; $n=6$) rats were used. The systolic blood pressure (SBP) of rats was recorded by the tail-cuff method connected to an electronic physiograph (PowerLab system). On the day of the experiment, the SBP of the rats that were obtained before oral administration of FA-I was noted as time 0. The rats were then fed by gavaging with 0, 50 or 100 mg/kg FA-I dissolved in distilled water (vehicle). The SBP of the rats were measured at 2, 4, 6, 8 and 24 hours after the oral treatment.

Results: The fraction, FA-I significantly ($p<0.001$) decreased the SBP in SHR rats in a dose-dependant manner after a single oral treatment. In addition, the drop in SBP was most significant ($p<0.01$) at 6 hours after the oral treatment when compared the vehicle group. However, the SBP of the rats returned to pretreatment level after 24 hours.

Conclusion: These results demonstrate that oral administrations of FA-I can decrease the BP of SHR with the highest effect being evident at 6 hours after treatment.

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APPENDIX A - SEPHADEX LH-20 GEL FILTRATION CHROMATOGRAPHY

About 80 g of dry Sephadex LH-20 gel were swelled in distilled water and then packed into a glass column with stopcock (2.5 cm, i.d. x 75 cm, height). Five grams of lyophilised FA dissolved in 5.0 ml of distilled water were applied to the column. The column was eluted with distilled water continuously at a flow rate of 0.5 ml/min by using a peristaltic pump. Caution was needed to avoid drying of the column. A fraction collector was used and each tube collected about 6.0 ml of fractions. The obtained fractions were then analysed by plotting on TLC aluminium plates coated with silica gel 60 F₂₅₄ (20 cm x 10 cm x 0.25 mm) with mobile phase being a combination of *n*-butanol : acetic acid : distilled water (B : A : W) (4 : 1 : 5 v/v, upper layer). The TLC plates were developed twice and viewed under UV light at 365 and 254 nm. The fractions that showed the same profile with the first fraction eluted were further confirmed by spraying with ninhydrin reagent before combining them together to obtain the FA-I fraction. The FA-I was then lyophilised and stored at -20°C for future use.

APPENDIX B - PREPARATIVE THIN LAYER CHROMATOGRAPHY

According to the study by Hoe *et al*, 2007, FA-I showed significant antihypertensive activity and yielded the highest amount, thus it was subjected to preparative TLC for further purification. Thin layer chromatography aluminium plates coated with silica gel 60 F₂₅₄ were used. The FA-I solution was applied to several spots arranged in a straight line with each spot separated 1 cm. The mobile phase was B : A : W (4 : 1 : 5, v/v, upper layer) and the TLC plates were developed twice. After development, strips of 1.0 cm width were cut from the center and edges of the TLC plates. The strips that cut out were sprayed with ninhydrin reagent and then compared with the unsprayed TLC plates to locate the positions of the FA-I subfractions. Each region that was corresponding to each compound on the plate was scrapped off and collected in separate vials. The compounds that were absorbed to the silica gel were desorbed by mixing them with methanol. The vials were then centrifugated at 3,000 x g for 20 min. This desorption process was repeated three times for maximum recovery of the compounds. The methanol solvent was then evaporated to obtain the compounds from each region in TLC plates.

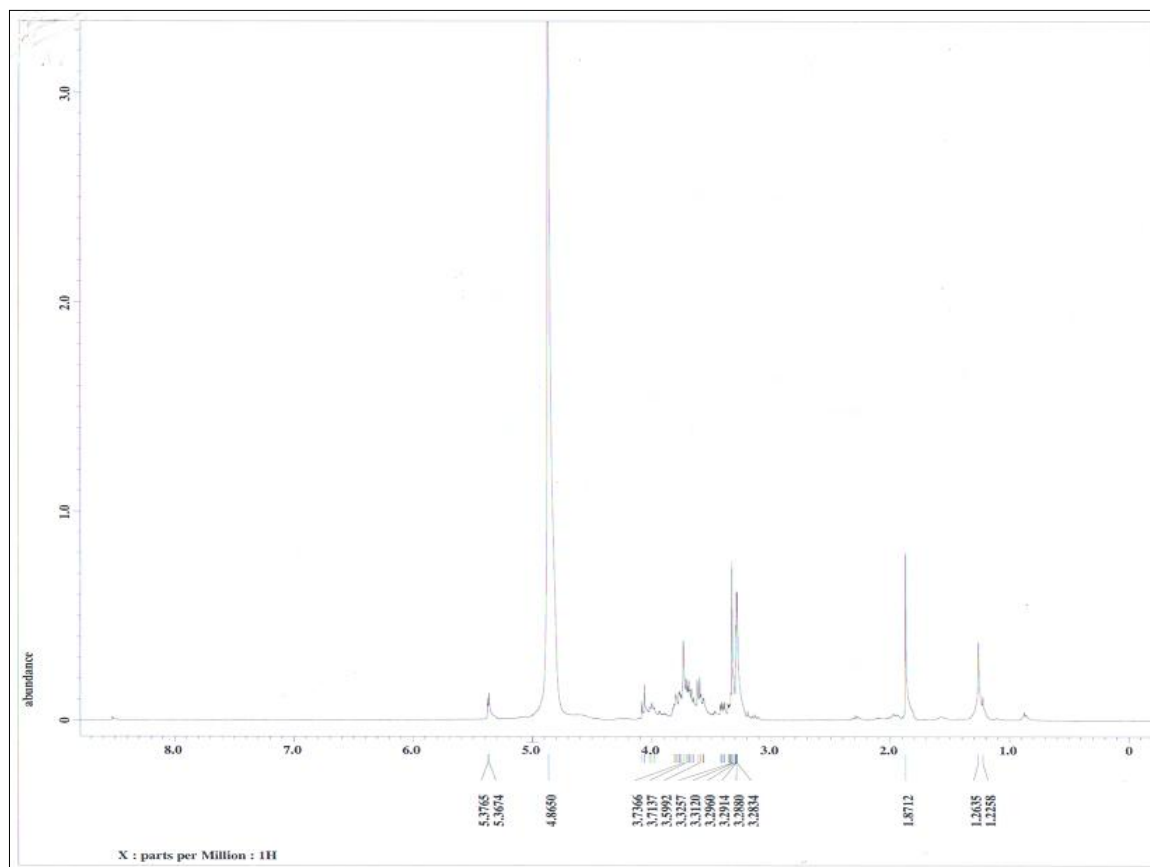
APPENDIX C - NINHYDRIN REAGENT

Ninhydrin reagent is commonly used to detect amino acids, amines and peptides. It reacts with amino acids and peptides to give a purple/red colour. In this study, it was prepared by dissolving 0.2 g of ninhydrin in 100 ml of acetone. After spraying the TLC plates with ninhydrin reagent, the plates were heated with hair dryer until colouration was seen. Care must be taken not to leave any fingerprint on TLC plates as ninhydrin is sensitive to fingerprint.

APPENDIX D - COMPOSITIONS OF KREBS-HENSELEIT SOLUTION

Materials	Composition (mM)
NaCl	118.0
KCl	4.70
CaCl ₂	2.5
MgSO ₄	1.2
KH ₂ PO ₄	1.2
NaHCO ₃	24.9
Glucose	11.1

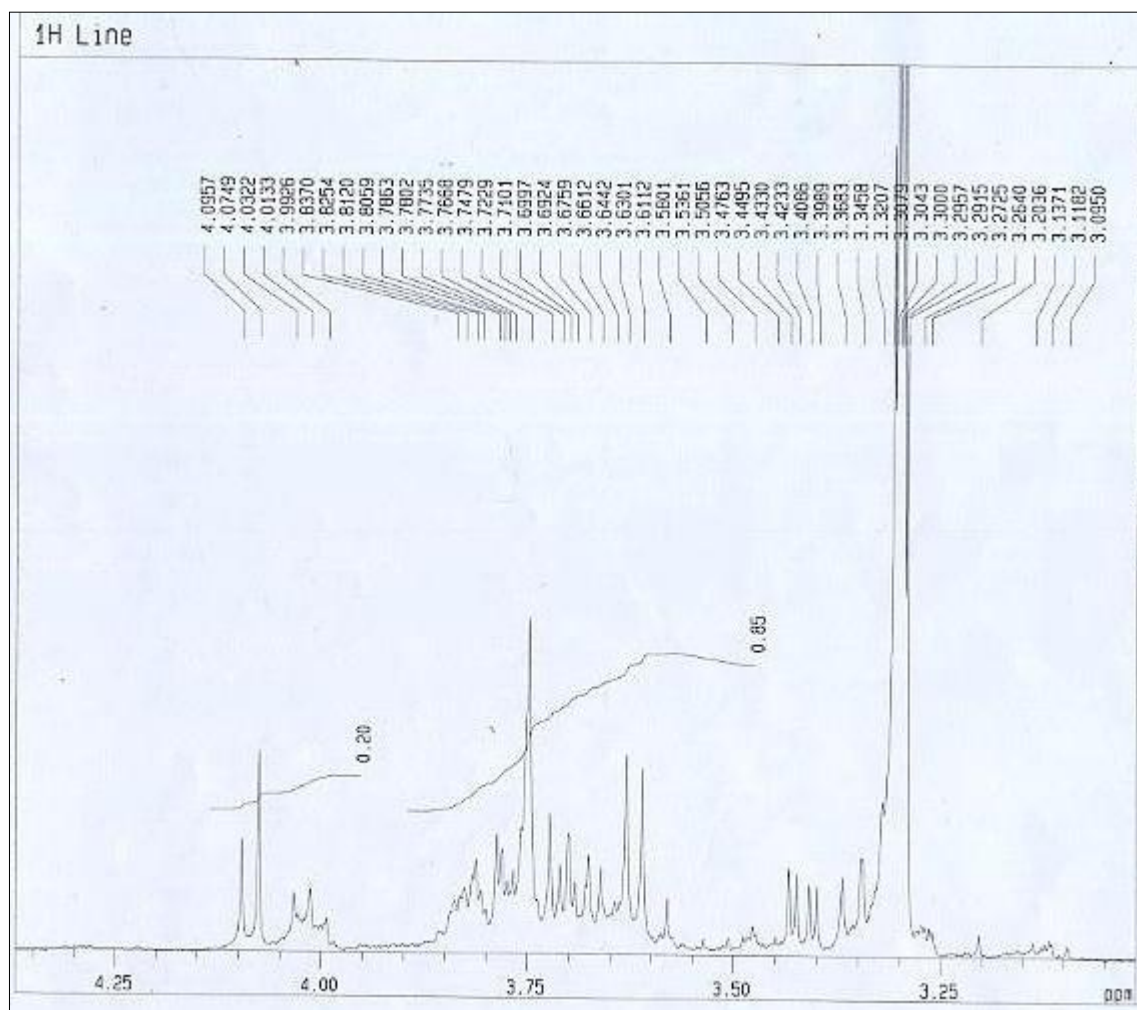
APPENDIX E - ^1H NUCLEAR MAGNETIC RESONANCE SPECTRUM (NMR) OF FA-1A AND THE TABULATED CHEMICAL SHIFTS



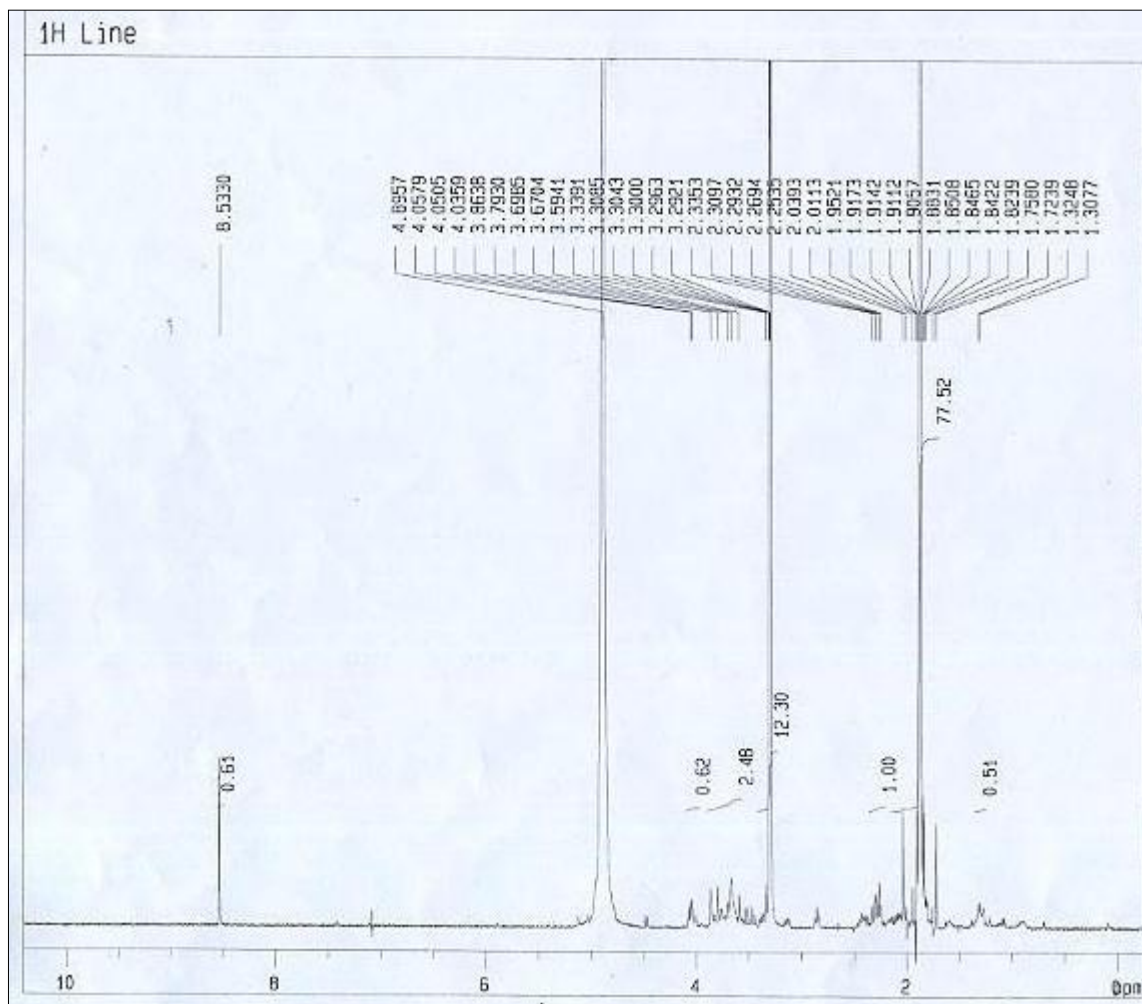
Hydrogen*	Chemical shift δ (ppm)
H1	5.38
H2	5.37
H3	3.73
H4	3.71
H5	3.6
H6	3.33
H7	3.31
H8	3.3
H9	3.29
H10	3.29
H11	3.28
H12	1.87
H13	1.26
H14	1.23

*Unassigned

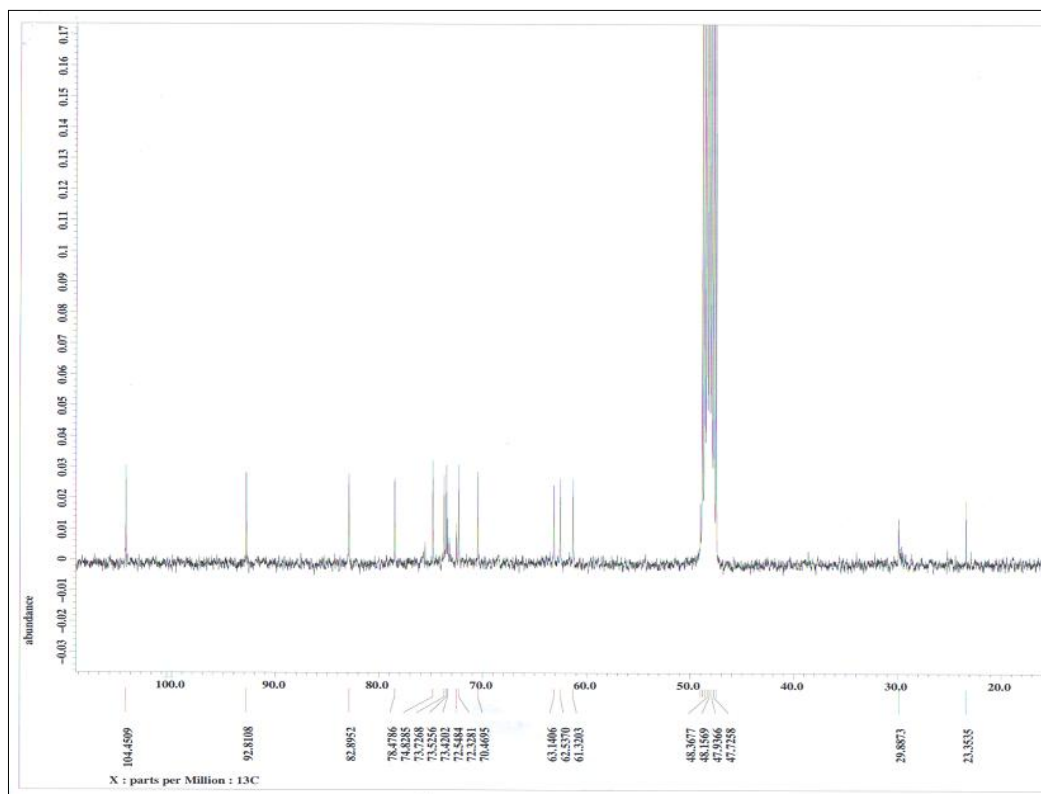
APPENDIX F - ^1H NUCLEAR MAGNETIC RESONANCE SPECTRUM (NMR) OF FA-IB



APPENDIX G - ^1H NUCLEAR MAGNETIC RESONANCE SPECTRUM (NMR) OF FA-IC



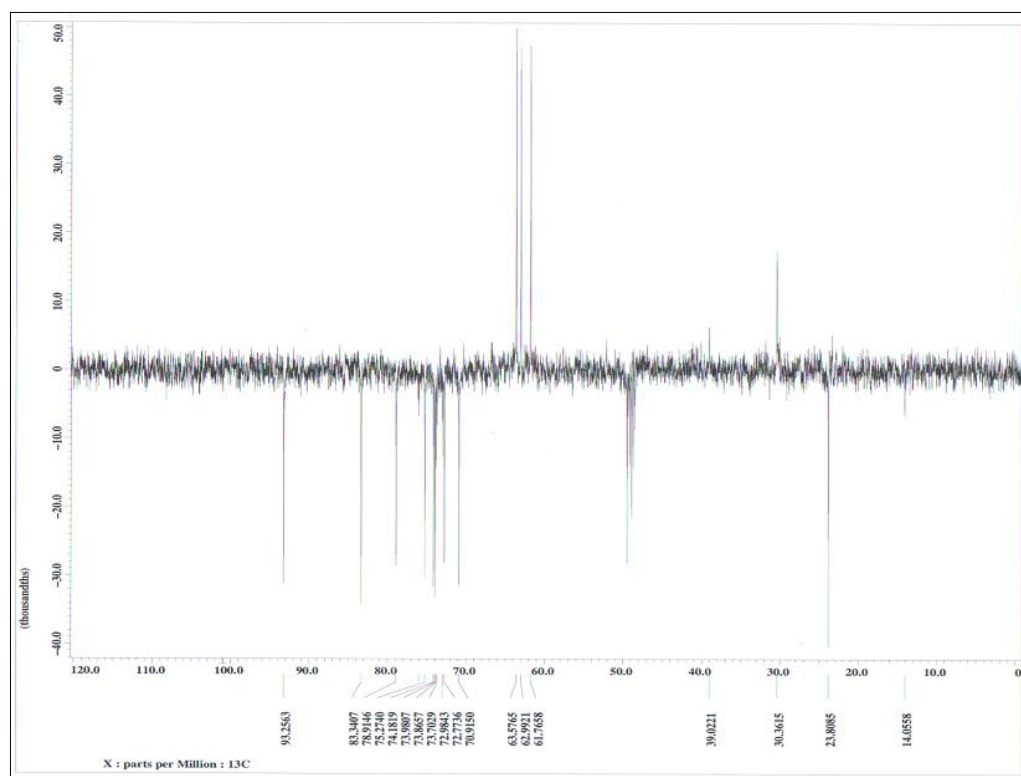
APPENDIX H - ^{13}C NUCLEAR MAGNETIC RESONANCE SPECTRUM (NMR) OF FA-1A AND THE TABULATED CHEMICAL SHIFTS



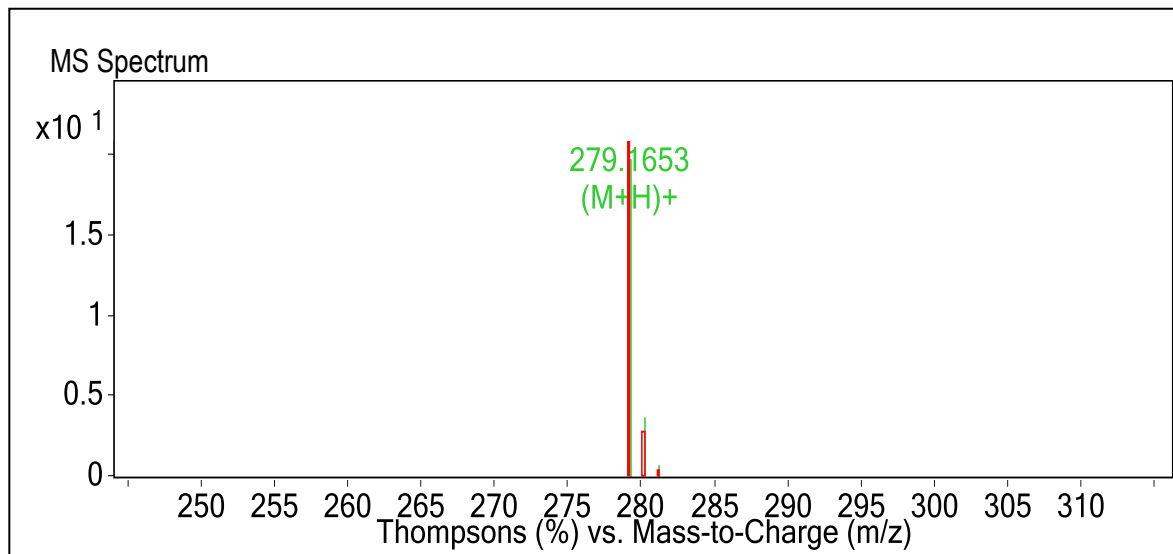
Carbon*	Chemical shift δ (ppm)
C1	104.45
C2	92.81
C3	82.9
C4	78.48
C5	74.83
C6	73.73
C7	73.53
C8	73.42
C9	72.55
C10	72.33
C11	70.47
C12	63.14
C13	62.54
C14	61.32
C15	29.89
C16	23.35

*Unassigned

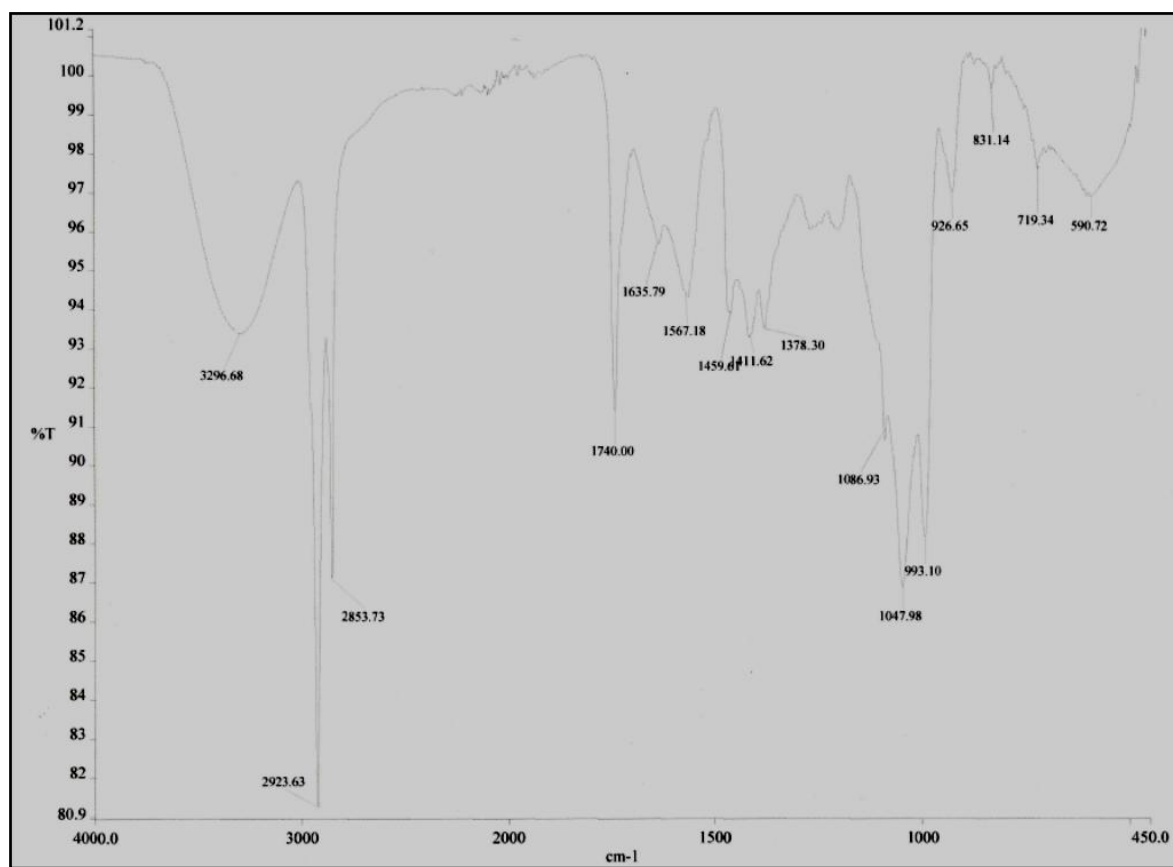
APPENDIX I - 2D DISTORTIONLESS ENHANCEMENT BY POLARISATION TRANSFER (DEPT) SPECTRUM OF FA-IA



APPENDIX J - LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS) SPECTRUM OF FA-IA



APPENDIX K - INFRARED SPECTRUM OF FA-IA



**APPENDIX L - SUGGESTED RANDOM COIL CHEMICAL SHIFTS (IN PPM)
FOR THE COMMON AMINO ACIDS**

Amino acid	¹ HN	¹³ C			¹³ CO
		¹ Hα	α	¹³ Cβ	
Cys (red)	8.32	4.55	58.2	28.0	174.6
Cys (oxd)	8.43	4.71	55.4	41.1	174.6
Asp	8.34	4.64	54.2	41.1	176.3
Glu	8.42	4.35	56.6	29.9	176.6
Phe	8.30	4.62	57.7	39.6	175.8
Gly	8.33	3.96	45.1	-	174.9
His	8.42	4.73	55.0	29.0	174.1
Ile	8.00	4.17	61.1	38.8	176.4
Lys	8.29	4.32	56.2	33.1	176.6
Leu	8.16	4.34	55.1	42.4	177.6
Met	8.28	4.48	55.4	32.9	176.3
Asn	8.40	4.74	53.1	38.9	175.2
Pro	-	4.42	63.3	32.1	177.3
Gln	8.32	4.34	55.7	29.4	176.0
Arg	8.23	4.34	56.0	30.9	176.3
Ser	8.31	4.47	58.3	63.8	174.6
Thr	8.15	4.35	61.8	69.8	174.7
Val	8.03	4.12	62.2	32.9	176.3
Trp	8.25	4.66	57.5	29.6	176.1

(Wishart and Nip, 1998, with slight modifications)